PURIFICATION AND PROPERTIES OF DPN-LINKED ISOCITRIC DEHYDROGENASE OF BOVINE HEART

by

Raymond Feng-chu Chen

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by

Raymond Feng-chu Chen

has been approved
August, 1963

Chairman, Supervisory Committee

Reader, Supervisory Committee

Reader, Supervisory Committee

Reader, Supervisory Committee

Reader, Supervisory Committee

Head, Major Department

Dean, Graduate School

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

There is general acceptance of the fact that the tricarboxylic acid cycle, first formulated by Krebs and Johnson (1) in 1937, is one of the major pathways for the oxidation of carbohydrate in living tissues. A considerable body of information has now accumulated on the various enzymes involved in the cycle, and progress has been made in the isolation and purification of these enzymes. Condensing enzyme (2) and fumarase (3) have now been crystallized. Although the other enzymes of the cycle have not been crystallized, a large number of studies have been done on each of them, with one possible exception. In the case of isocitric dehydrogenase, many studies have been done on the TPN-linked[1] enzyme from a number of tissues; but relatively little is known concerning the DPN-linked isocitric dehydrogenase. The latter enzyme has been neglected although it was first found in yeast by Kornberg and Pricer (4) in 1951 and later in animal tissues in 1954 by Plaut and Sung (5).

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[1] The abbreviations of nucleotides used are those approved by the Journal of Biological Chemistry. In addition, DEAE and CM-cellulose are diethylaminoethyl- and carboxymethyl-cellulose. ADPR is adenosine diphosphate ribose. An example of the abbreviations used for pyridine nucleotide analogues is acetylpyridine-DPN$^+$, the 3-acetylpyridine analogue of DPN$^+$. Tris is tris(hydroxymethyl)aminomethane. EDTA is ethylenediaminetetraacetate.

The present Thesis will present experimental data on the purification and properties of the DPN-linked isocitric dehydrogenase of beef heart mitochondria. It seems apparent that up to the present, this enzyme has been overlooked because other workers have assumed that the well-studied and abundantly available TPN-linked isocitric dehydrogenase is the enzyme involved in the tricarboxylic acid cycle. It is worthwhile to review relevant aspects of isocitrate metabolism in order that the present studies may be viewed in the proper perspective.

I. ISOCITRATE OXIDATION

Historical Note. Detailed studies on the metabolic fate of isocitrate in biological tissues may be said to have been stimulated by the work of Martius and Knoop, when these authors in 1937 investigated the conversion of citric acid to succinate (6,7). They suggested a pathway whereby citrate was converted to aconitate, isocitrate, and oxalosuccinate before decarboxylation to yield α-ketoglutarate. Martius (8) subsequently isolated isocitrate from animal tissues and postulated that after isocitrate was oxidized to oxalosuccinate, the latter would decarboxylate spontaneously to yield α-ketoglutarate and CO_2. At about this time, Krebs and Johnson (1) showed the formation of citrate from oxalacetate and pyruvate and put forth the concept of a "citric acid cycle" as a metabolic route for the terminal oxidation of carbohydrates. It was Adler et al. (9) in 1939 who published the first

detailed study of an enzyme catalyzing the oxidation of isocitrate and showed that this enzyme was separate from the system catalyzing the conversion of citrate to isocitrate. These workers isolated an isocitrate dehydrogenase from various animal organs and showed that it was dependent on TPN^+ and a metal ion, preferably Mn^{++}. The enzyme described by Adler et al. (9) remained for many years the only recognized isocitric dehydrogenase in animal tissues. Pig heart acetone powders served as a good source of this enzyme, but it was present also in high amounts in liver, kidney, and adrenal gland (9). Many studies have now been done on this enzyme, and some of its features should be pointed out.

TPN-Linked Isocitric Dehydrogenase. The reaction catalyzed by the enzyme can be written as follows:

threo-D_s-isocitrate[2] α-ketoglutarate

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[2]The correct structural formula for the naturally occurring isocitrate of most living tissues was only recently established by Kaneko et al. (10,11) who employed degradative and synthetic organic chemical techniques. The structure was confirmed by Patterson et al. (12) by X-ray crystallography. The nomenclature used above is that suggested by Vickery (13).

As written, the reaction is a concerted oxidative decarboxylation of isocitrate. The original suggestion that oxalosuccinate, $HOOCCOCH(COOH)CH_2COOH$, was an intermediate was apparently supported at one time by the finding that animal tissues contained enzyme(s) which could reduce oxalosuccinate to isocitrate ("oxalosuccinic reductase") or decarboxylate oxalosuccinate to α-ketoglutarate ("oxalosuccinic decarboxylase") (14,15). It was not clear for some time whether these enzymic activities belonged to TPN-linked isocitric dehydrogenase or to other enzymes. Moyle and Dixon (16,17) showed that extensive purification of TPN-linked isocitric dehydrogenase of pig heart did not separate the enzymic activities capable of acting on oxalosuccinate. Purification of the enzyme by Siebert et al. (18) until it was virtually homogeneous in the ultracentrifuge yielded an enzyme preparation with isocitric dehydrogenase, oxalosuccinic decarboxylase, and oxalosuccinic reductase activities. Furthermore, these activities were present in about the same proportions throughout the purification. Thus, all of these activities were exhibited by a single protein. Attempts to isolate oxalosuccinate as a free intermediate were fruitless and it was apparent from experiments using isotope-labeled compounds that oxalosuccinate could occur as an enzyme-bound intermediate, but definitely was not an obligatory free intermediate (19).

Physical studies of the pig heart enzyme revealed that it had a molecular weight of 61,000 and a turnover number of 3,500 moles of TPNH formed per mole per minute (18). Sufficient enzyme was

obtained to permit study both by ultracentrifugation and by electrophoresis. However, the enzyme was relatively unstable in solutions of low ionic strength and apparently became denatured on electrophoresis (18). Stability could be achieved by suspension in concentrated ammonium sulfate solutions (0.2 to 0.4 saturated) (20).

The intracellular location of this enzyme in heart was studied by Plaut and Plaut (21) who found only 5% of the total activity of the cell present in the mitochondrial fraction, the bulk of the remaining activity being in the soluble fraction. Hogeboom and Schneider (22), in experiments with mouse liver, found the mitochondrial fraction to contain 12% of the total activity, while the supernatant fractions had 82%. These authors considered it possible that the activity in the mitochondrial fraction could have been due to adsorption; i.e., that the mitochondrial activity was an artefact. Ernster and Navazio (23) used an indirect assay for the enzyme in rat liver and decided that the ratio of mitochondrial to cytoplasmic activities was 1:9. However, these authors (24) felt that part of the TPN-linked isocitric dehydrogenase was actually intramitochondrial rather than adsorbed, because the TPN-dependent isocitrate oxidase activity of mitochondria became maximal only after an ageing procedure which presumably made the mitochondrial membrane more permeable to isocitrate. Shepherd and Kalnitsky (25) found that TPN-linked isocitric dehydrogenase of rabbit liver was localized in the supernatant fraction, but in cerebral cortex, the mitochondrial fraction contained about 50% of

the total activity. Shepherd (26) considers all the TPN-linked isocitric dehydrogenase of cerebral cortex to be mitochondrial and says that the enzyme which appears in the soluble fraction has probably "leaked out" of the mitochondria. However, Schneider (27) feels that the reverse situation is more probable; viz., that adsorption of supernatant enzyme onto mitochondria has occurred. In most tissues, however, there does seem to be general agreement that the TPN-linked isocitric dehydrogenase is extramitochondrial (27).

DPN-Linked Isocitric Dehydrogenase. The first demonstration of a DPN-linked isocitric dehydrogenase came in 1951 when Kornberg and Pricer (4) found such an enzyme in baker's and ale yeast. They showed that this enzyme could be separated from the TPN-linked isocitric dehydrogenase also present in autolysates. The DPN-linked enzyme precipitated at lower concentrations of ammonium sulfate than did the TPN-specific enzyme. It was easier to isolate the TPN-linked enzyme free from the DPN-enzyme, however, since the latter was less stable. The DPN-linked enzyme apparently catalyzed the same over-all oxidative decarboxylation reaction as did the TPN-linked enzyme except with a different coenzyme. Mn^{++} was found to be more effective than Mg^{++} as an activator. Kornberg and Pricer found that the DPN-linked enzyme would neither reduce nor decarboxylate oxalosuccinate and would not catalyze the reductive carboxylation of α-ketoglutarate to yield isocitrate. A further observation was that DPN-linked isocitric dehydrogenase

seemed to be totally inactivated by charcoal treatment, suggesting that a specific cofactor was required. This was found to be 5'-AMP. A specific 5'-AMP deaminase could replace charcoal for the inactivation. These authors found that the cofactor was tightly bound to the enzyme even after purification by repeated ammonium sulfate fractionations and calcium phosphate gel adsorption and elution. In experiments with the charcoal-treated enzyme, the authors noted that half-maximal reactivation could be achieved with 9×10^{-6} M 5'-AMP. ADP seemed able to replace the 5'-AMP requirement to some extent. The possibility that 5'-AMP might be a contaminant of ADP seemed to be ruled out by prior incubation of the ADP solution with adenylic deaminase, and 5'-AMP could not satisfy the nucleotide requirement. No mention was made however, of the possibility that adenylic deaminase might be inhibited by ADP. Other compounds which failed to activate the yeast DPN-specific isocitric dehydrogenase were adenosine, ATP, 2'-AMP, and 3'-AMP. Because of the above features of yeast DPN-linked isocitric dehydrogenase, the reaction which is catalyzed may be written as follows:

$$\underline{\text{threo-D}_s}\text{-isocitrate} + \text{DPN}^+ \xrightarrow[5'\text{-AMP}]{Mn^{++}} \alpha\text{-ketoglutarate} + CO_2 + \text{DPNH} + H^+$$

The occurrence of DPN-linked isocitric dehydrogenase in animal tissues was reported by Plaut and Sung (5) in 1954. The enzyme was looked for in cardiac mitochondria because prior studies on the metabolism of such tissues showed that citrate oxidation could be

stimulated by adding DPN^+ to the incubation mixture (21). This observation suggested that a DPN-linked isocitric dehydrogenase might be present, whose activity was being limited by a lack of DPN^+. The DPN-specific enzyme was separated from the TPN-linked enzyme in the following tissues: guinea pig and beef heart, and pigeon breast muscle (5). The animal enzyme was unlike the yeast enzyme in that 5'-AMP did not seem to be required. However, the enzymes were similar in the following respects: 1. Mn^{++} was more active than Mg^{++}. 2. Oxalosuccinate was not a substrate. 3. The reversal of the oxidative decarboxylation of isocitrate could not be achieved. Further details of this enzyme are given in the last section of this Introduction.

A subsequent study by Neufeld et al. (28) described the isolation of DPN-linked isocitric dehydrogenase from pig heart. Later, Sung and Hsü (29), studying Krebs cycle enzymes in human placentas, isolated the DPN-linked isocitric dehydrogenase and separated it from the corresponding TPN-specific enzyme, which was present in 20-fold greater amounts (in terms of activity) in extracts of mitochondrial acetone powders. The activity per mg. of protein found in extracts of placental mitochondrial acetone powders was 10-20 times lower than found previously in extracts of similar powders prepared from beef heart (5). It was noted that estradiol in a concentration of 8×10^{-6} M had no effect on the activity of the DPN-linked enzyme.

Apparently, the only other sources from which the DPN-linked

isocitric dehydrogenase had been isolated are Aspergillus niger (30) and pea seedlings (31). Ramakrishnan and Martin (30) found that the enzyme from A. niger, like the yeast enzyme, was dependent on 5'-AMP for activity. In addition, it was found that dialysis of a phosphate-containing enzyme solution against Tris buffer markedly reduced activity, which was restored by adding phosphate. This is the only DPN-linked isocitric dehydrogenase which has been reported to be activated by phosphate. The pea seedling enzyme was not studied in terms of nucleotide requirements. However, both the A. niger enzyme and the pea enzyme failed to catalyze the reductive carboxylation of α-ketoglutarate.

It is evident that the list of papers concerning the isolation of DPN-linked isocitric dehydrogenase from various tissues is quite brief. It is possible that this is due to the fact that the enzymes from yeast, heart, placenta, and A. niger are quite unstable and the amounts small, so that detailed studies are not easily performed. In addition, most tissues contain the TPN-linked isocitric dehydrogenase in far greater amounts, so that a superficial view may be taken that the DPN-linked enzyme is relatively unimportant. Some idea of the relative proportions of these two enzymes in the cell may be obtained by the following considerations.

Only 5-12% of the total amount of TPN-linked isocitric dehydrogenase activity of the cell is associated with mitochondria (21,22,23), while all of the DPN-linked isocitric dehydrogenase seems to be mitochondrial (5,23,29). However, extracts of mitochondrial acetone

powders still yield 20-30 times more TPN- than DPN-linked isocitric dehydrogenase activity (5). Thus, of the total isocitric dehydrogenase activity of the cell, considerably less than 1% would seem to be DPN-linked. However, such calculations assume that optimal *in vitro* assay procedures reflect the *in situ* activities of the enzymes accurately, and this assumption may be incorrect. Further considerations which have a bearing on the assay of the enzymes *in vitro* and *in vivo* are indicated below. One should also realize that the amount of a given enzyme in the cell may not reflect the *importance* of that enzyme in metabolism.

Controversy Concerning the Pathway of Isocitrate Oxidation. Since there are two known isocitric dehydrogenases, the question arises as to whether they are involved in the same or different metabolic pathways. Isocitric dehydrogenase is needed in the Krebs cycle, but the enzyme might also be needed to generate reduced pyridine nucleotide for biosynthetic reactions such as fatty acid synthesis, steroid hydroxylation, squalene synthesis, and the like. Attempts have been made to distinguish which isocitric dehydrogenase is involved in mitochondrial oxidation of isocitrate.

It is well established that the citric acid cycle is operative in isolated mitochondria (27,32) from a variety of sources. Therefore, mitochondria have been used to study the question of whether isocitrate is oxidized in the Krebs cycle via the TPN- or the DPN-specific isocitric dehydrogenase. However, if one uses suspensions of intact mitochondria and tries to detect isocitric dehydrogenase

activity by the change in optical density at 340 mμ, certain practical and theoretical difficulties arise. For example, in the case of heart, no reduction of DPN^{+} in the presence of isocitrate can be demonstrated in suspensions of mitochondria even though heart mitochondrial acetone powders contain DPN-linked isocitric dehydrogenase (5). One reason for the discrepancy may be that isocitrate does not penetrate into the mitochondria under certain conditions. Indeed, Johnson and Lardy (33) produced marked effects on citrate and isocitrate oxidation in liver mitochondria by changing the tonicity of the suspending medium. Other evidence exists that citrate and isocitrate may penetrate into such mitochondria only with difficulty. Plaut and Plaut (21) showed that added tricarboxylic acids were oxidized at very slow rates by heart mitochondria although acetate was apparently metabolized via the Krebs cycle (21,34) without difficulty. Lester et al. (35,36) also found that added citrate and isocitrate was not oxidized by beef heart mitochondria unless the membranes were damaged by treatment with detergents. Montgomery and Webb (37), however, felt that permeability was not the limiting factor in citrate oxidation by heart mitochondria, since lowering of the pH of the suspending medium to allow greater penetration resulted in depression of oxidation. Other interpretations of their results are possible, however. Another problem which arises stems from the fact that the "DPNH-oxidase" activity of mitochondria is generally quite high (27), so that DPNH, once formed, would not accumulate, but would be

oxidized immediately either along the electron transport system, or, if endogenous substrate were present, could be reoxidized via a competing enzymic reaction, such as the reduction of α-ketoglutarate to glutamate if ammonia were present. The effect of "DPNH oxidase" activity would be more marked if the enzymic reaction under investigation occurred relatively slowly, as seems to be the case with DPN-linked isocitric dehydrogenase. Furthermore, if one tries to detect reduction of added DPN^+ or TPN^+, the question arises as to whether the nucleotides can penetrate within the mitochondria or whether any reduction observed is produced by adsorbed, extramitochondrial enzymes.

In spite of these difficulties, the problem of which isocitric dehydrogenase partakes in the Krebs cycle has been considered by a number of workers using intact mitochondria. If mitochondria are incubated with isocitrate in the absence of any other substrate, the amount of oxygen taken up will be a measure of the "isocitric oxidase" activity. Using mouse liver, Hogeboom and Schneider (22) found that the isocitric oxidase activity was entirely in the mitochondria, a finding that was not unexpected in view of the fact that only the mitochondria contain the terminal electron transport system. Ernster and Navazio (23,24) found that if liver mitochondria are incubated in 0.25 M sucrose- .05 M phosphate buffer for 30 minutes, the isocitrate oxidase activity is lost. This treatment was found by Siekevitz and Potter (38) to produce a loss of a material from mitochondria absorbing at 260 mμ. The data of Hunter

and Ford (39) indicate that this material is at least partly DPN^+, since such depleted mitochondria failed to carry out DPN-linked oxidations, and such oxidations could be restored by adding DPN^+. It would appear that TPN^+ is also depleted from the mitochondria, since Ernster and Glasky (40) were unable to obtain reduction of 2,6-dichlorophenol-indophenol with isocitrate as substrate, unless TPN^+ or DPN^+ were added. Since DPN^+ restored the isocitric oxidase activity level to that of undepleted mitochondria, Ernster and Navazio (23,24) concluded that a DPN-linked enzyme was responsible for the initial oxidation of isocitrate, especially since DPN^+ plus TPN^+ did not seem more effective than DPN^+ alone, and TPN^+ alone seemed to have a negligible effect. These authors did not dispute the fact that the TPN-linked isocitric dehydrogenase was present in their preparations and more active than the DPN-linked enzyme (24). The reason, therefore, that TPN^+ failed to restore isocitric oxidase activity fully was, therefore, attributed to the inability of the electron transport system to use the TPNH. By itself, this observation would not rule out the possibility that undepleted mitochondria could oxidize isocitrate via TPN^+ and that the TPNH could be converted to DPNH via pyridine nucleotide transhydrogenase. However, there seemed to be too little transhydrogenase activity in rat liver mitochondria to account for all the isocitric oxidase activity (43).

Kaplan's group (41), on the other hand, have never admitted that DPN-linked isocitric dehydrogenase exists at all in rat liver

mitochondria. Stein et al. (41) could not detect DPN-dependent reduction of 2,6-dichlorophenolindophenol by isocitrate in nucleotide-depleted rat liver mitochondria. These workers, therefore, favor a pathway whereby isocitrate is oxidized by the TPN-linked isocitric dehydrogenase and the TPNH which is produced is then converted to DPNH by transhydrogenase. These workers found some 40-50 times as much transhydrogenase activity as did Ernster and Navazio (23). Purvis (42) confirmed the findings of Stein et al. (41) in experiments employing nucleotide-depleted mitochondria of rat liver and rat heart: no DPN-linked isocitric dehydrogenase activity could be detected by the use of 2,6-dichlorophenolindophenol. Transhydrogenase was noted to be extremely active. However, both Stein et al. (41) and Purvis (42) used an "improved" transhydrogenase assay in which the 3-acetylpyridine analogue of DPN^+ was the hydrogen acceptor. Ernster (43), in a rebuttal, considers this assay capable of giving falsely high values for transhydrogenase because 3-acetylpyridine-DPN^+ has a greater reduction potential than does DPN^+. This seems to be a valid criticism: Spiegel and Drysdale (44) subsequently showed that transhydrogenation from DPNH to 3-acetylpyridine-DPN^+ occurred non-enzymically. Reaffirming previous results, Ernster and Glasky (40) found that DPN^+ fully restored both citrate and isocitrate oxidation, and that $DPN^+ + TPN^+$ was not more effective.

The obviously conflicting results cited above have been reviewed by Plaut (45) who suggests that subtle differences in

experimental technique may be an explanation.

It is difficult to correlate the experiments done with intact mitochondria with work done on purified enzymes. Although Plaut and Sung (5) assayed for DPN-linked isocitric dehydrogenase in extracts of rat liver mitochondrial acetone powder, very little activity was found. Ernster and Navazio (24), however, using dye reduction techniques, decided that the DPN-linked enzyme was highly active in liver mitochondria. Their data indicate that the ratio of TPN-specific to DPN-specific isocitric dehydrogenase activities in this tissue is only 4. It seems surprising that Purvis (42) could not demonstrate the DPN-linked isocitric dehydrogenase in nucleotide-depleted rat heart mitochondria with 2,6-dichlorophenolindophenol as electron acceptor. The enzyme has been isolated from heart mitochondria of cattle, pigeon, and guinea pig (5) as well as swine (28), and, therefore, might be expected to be in rat heart as well. For this reason, the report of failure to demonstrate the DPN-linked isocitric dehydrogenase in rat <u>liver</u> mitochondria (42) using the <u>same</u> techniques is of dubious significance.

Besides liver and heart, certain other tissues have been studied with regard to the pathway of isocitrate oxidation. Grant and Mongkolkul (46), using ox adrenal cortical mitochondria, found that the steroid 11-β-hydroxylase system was only weakly stimulated by citrate although it was known that this system was dependent on TPNH and molecular oxygen. Because of these results, the authors suspected that TPN-linked isocitric dehydrogenase could not be very

active in these mitochondria. They subsequently performed experiments with nucleotide-depleted adrenal cortical mitochondria and found that isocitrate reduction of 2,6-dichlorophenolindophenol was restored by adding back DPN^+, but not TPN^+ (47). The possibility that transhydrogenase activity could be responsible for this was ruled out by direct measurements. Oxygen uptake with citrate as substrate was also restored by DPN^+ but not with TPN^+. Thus, in adrenal mitochondria, the evidence points to a DPN-linked isocitrate oxidation pathway. Similar results have now been found by Hawtrey and Silk (48) who studied the pathway of isocitrate oxidation in mitochondria of Ehrlich ascites tumor cells. Mitochondria were obtained after rupture of the cells by osmotic shock (49). Measurements of enzyme activity using 2,6-dichlorophenolindophenol as electron acceptor indicated that there was 3 to 4 times as much DPN- as TPN-specific isocitric dehydrogenase activity. Tager (50) has reported that mitochondria of ripe papaya fruit and of other plants seem to contain predominantly the DPN-linked isocitric dehydrogenase; the same results were obtained with 2,6-dichlorophenolindophenol, ferricytochrome c, or O_2 as electron acceptor. TPN-linked isocitric dehydrogenase was also present but it was extramitochondrial.

It is clear that the problem of the pathway of isocitrate oxidation is still unsolved. Aside from differences in techniques used by different laboratories, it is possible that various tissues may employ different pathways for the oxidation of isocitrate. In

addition, mitochondrial preparations from various sources may be contaminated by adsorbed enzymes to a different degree. A corollary of this possibility would be that different tissues may exhibit marked variations in viability of the truly intramitochondrial enzymes under identical preparative conditions.

In view of the uncertainty about the pathway of isocitrate oxidation, it seemed fitting that further studies on the isolated DPN-linked isocitrate dehydrogenase be carried out. Even though only one detailed study of the mammalian enzyme had been reported (5), certain facts had already come to light which would have a bearing on studies using intact mitochondria. For instance, the DPN-linked isocitric dehydrogenase was relatively unstable and would probably be damaged more easily than the TPN-linked enzyme. Furthermore, the DPN-specific enzyme was inhibited by cyanide (5) which is often used to block cytochrome oxidase in experiments with intact mitochondria. It had also been found that ATP was a strong inhibitor of the DPN-linked enzyme (5), and under aerobic conditions, mitochondria could well contain high concentrations of ATP. It was felt that further study of the enzyme might reveal other features relevant to its assay in cells and subcellular fractions.

II. PREVIOUS WORK ON DPN-LINKED ISOCITRIC DEHYDROGENASE OF BOVINE HEART

The only detailed report on this enzyme which has appeared to date is that of Plaut and Sung (5). Aside from showing that the

beef heart enzyme catalyzed an apparently irreversible reaction and was activated by Mn^{++} more than by Mg^{++}, this paper also surveyed the occurrence of DPN-linked isocitric dehydrogenase in a variety of animal tissues. The first source with which the authors obtained unequivocal evidence for the occurrence of the enzyme was guinea pig mitochondrial acetone powder. The DPN-linked enzyme from this source was partially separated from the TPN-specific isocitric dehydrogenase by simple ammonium sulfate fractionation. The former enzyme precipitated between 0.4 and 0.5 saturation, whereas the latter was found in the material precipitating between 0.6-0.8 saturation. The two enzymes from beef heart and pigeon breast muscle were also separated. Considerable DPN-linked isocitric dehydrogenase activity was found in extracts of acetone powders of pigeon heart and rat kidney mitochondria, but rat liver mitochondrial acetone powder seemed to contain very little activity.

The specific activity of extracts of guinea pig heart mitochondrial acetone powder was about ten times higher than obtained if whole heart acetone powder was used. Thus, the enzyme appeared to be wholly mitochondrial.

Studies on the DPN-linked enzyme from beef heart (a convenient source for larger quantities of enzyme) showed that TPN^+ and NMN^+ failed to react with or activate the enzyme. ATP "almost completely" inhibited the enzyme at a concentration of 0.002 M. Only threo-D_s-isocitrate would serve as substrate. The following were not acted

upon: threo-L_s-isocitrate, citrate, cis-aconitate, oxalosuccinate, dl-β-hydroxybutyrate, d- or l-α-hydroxyglutarate, glutaconate, or malate (51).

It was noted that the enzyme was quite unstable, so that preparations used for kinetic studies had to be made up daily. Even the acetone powder, when stored in the freezer, slowly lost DPN-linked isocitric dehydrogenase activity, whereas the corresponding TPN-linked enzyme activity did not decrease. Extracts of such mitochondrial acetone powder in 0.01 M potassium phosphate buffer, pH 6.5, contained about thirty times more TPN-specific than DPN-specific isocitric dehydrogenase activity. Simple ammonium sulfate fractionation yielded enzyme sufficiently clean for most of the kinetic measurements, but a more elaborate procedure involving calcium phosphate gel adsorption, ammonium sulfate fractionation, and chromatography on a starch-Celite column was devised for the highest purity. The TPN-linked enzyme was still detectable after a 40-50 fold purification of the original extract. The procedures for making the acetone powder and for purification of the DPN-specific enzyme were later summarized (51).

The pH optimum of the beef heart enzyme appeared to be fairly sharp and occurred at pH 6.5 in contrast to a broad pH dependency curve for the TPN-linked enzyme which had an optimum at about pH 7.8. K_M values for DPN^+ and isocitrate were about 6×10^{-5} M and

4.5×10^{-4} M, respectively.

Carsiotis (52) has done a limited number of inhibition studies on the DPN-linked isocitric dehydrogenase of beef heart, and his results will be mentioned in the subsequent chapter.

CHAPTER II

THE EFFECT OF CERTAIN NUCLEOTIDES ON DPN-LINKED ISOCITRIC DEHYDROGENASE

A systematic survey of the effect of a number of nucleotides os DPN-linked isocitric dehydrogenase was undertaken for several reasons. First, it was already known that the enzyme was inhibited by certain nucleotides. Since ATP seemed to inhibit the oxidation of citrate in heart mitochondria (21), Plaut and Sung (5) tested this compound on DPN-linked isocitric dehydrogenase and found ATP to be a strong inhibitor. Subsequently, Carsiotis (52) confirmed these results, noting that 100% inhibition was achieved at a concentration of nucleotide of 0.002 M. He also found that 2'-AMP inhibited the enzyme, although only weakly, since 0.015 M nucleotide was required for 50% inhibition. By increasing the DPN^+ concentration from 3.3×10^{-4} M to 3.3×10^{-3} M, Carsiotis (52) was able to reverse the inhibition by 2'-AMP completely. This finding suggested that 2'-AMP competed with DPN^+ for the same site on the enzyme. Second, DPN-linked isocitric dehydrogenases from both yeast and A. niger require a nucleotide, 5'-AMP, for maximal activity (4,30). Although the enzyme of heart did not seem to require 5'-AMP (5), it remained possible that other nucleotides might have some effect. Third, quite early in the

present studies, it was noted that the rate of DPNH formation decreased markedly with time, suggesting that the enzyme was being inhibited by the nucleotide, DPNH.

In the present studies, the nature of the inhibition by these and other nucleotides has been investigated. In addition, it has been discovered that the DPN-linked isocitric dehydrogenase of bovine heart is markedly stimulated by ADP. The characteristics of the ADP stimulation have been investigated.

I. MATERIALS AND METHODS

Reagents. Water was distilled and deionized before use. There was less than 1 ppm of electrolyte, determined as NaCl. Most solutions for kinetic studies were also redistilled following deionization.

The following compounds were obtained from the Sigma Chemical Co., St. Louis, Mo.: DPN^+, α-DPN^+ (the isomer of DPN^+ in which the nicotinamide riboside linkage has the α-configuration), DPNH, TPN^+, $TPNH^+$, 5'-AMP, ADP, dADP, ATP, ADPR, 3',5'-cyclic-AMP, ADP, ITP, GDP, GTP, UMP, UDP, CDP, CTP, riboflavin, riboflavin 5'-phosphate, and FAD. TPNH was also obtained from California Corporation for Biochemical Research, Los Angeles, California. Other preparations of TPNH were prepared from TPN^+ as indicated in the text. Adenine, adenosine, 2'-AMP, and 3'-AMP were obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y. dGMP and dAMP were purchased from California Corporation for Biochemical

Research. The above nucleotides, as 0.01 M aqueous solutions, were neutralized with KOH before use, except for solutions of the reduced pyridine nucleotides which were dissolved in 0.10 M $NaHCO_3$. For assay purposes, the following millimolar extinction coefficients (liter millimole^{-1}) were used (53): 6.22 for DPNH and TPNH at 340 mμ; 15.4 for adenine-containing nucleotides at 259 mμ; 12.2 for hypoxanthine-containing nucleotides at 262 mμ; 13.7 for guanine-containing nucleotides at 252 mμ; and 13.0 for cytosine-containing nucleotides at 280 mμ. NMN^+, acetylpyridine-DPN^+, deamino-DPN^+, thionicotinamide-DPN^+, and pyridinealdehyde-DPN^+ were obtained from the Pabst Laboratories, Milwaukee, Wisc.

Threo-D_sL_s-isocitric acid lactone from the California Corporation for Biochemical Research was hydrolyzed by boiling at 95° at pH 10 for 20 minutes and neutralized with HCl before use. Enzymic assay with TPN-linked isocitric dehydrogenase (20) indicated that the isocitrate was 99-100% pure. Throughout these studies, the racemic mixture of isocitrate was used, although the results are expressed in terms of the single active isomer, threo-D_s-isocitrate, unless otherwise stated. α-Ketoglutaric acid was obtained from the Aldrich Chemical Co. and the Matheson, Coleman and Bell Co. and purified by recrystallization from ether and benzene. The barium salt of glucose 6-phosphate heptahydrate was purchased from the Sigma Chemical Company and converted to a solution of the potassium salt before use (54).

ADP^{32}, with label in the terminal phosphate group, was a gift

of Dr. M. Chiga. The compound had been prepared by means of rat liver mitochondria and labeled inorganic phosphate (55).

Crystalline liver L-glutamic dehydrogenase (Type I, ammonium sulfate suspension) and yeast glucose 6-phosphate dehydrogenase (Type V) were purchased from the Sigma Chemical Company.

DEAE-cellulose from the Brown Co., Berlin, N.H., 0.9 meq/g, was washed by the procedure of Kaziro et al. (56) before equilibration with buffer and packing into columns. In this procedure, the cellulose resin is repeatedly suspended in 1.0 N NaOH until the washings are colorless. After washing with water, the DEAE-cellulose is then suspended in 0.5 N HCl and filtered through a large Buchner funnel. The resin is then washed with water until chloride-free (as judged by testing with $AgNO_3$). The cellulose is then suspended in 0.005 M potassium phosphate buffer and the fines removed.

CM-cellulose with a capacity of 0.7 meq/gm from the Brown Co. was similarly washed before use.

Ammonium sulfate solutions were adjusted to pH 7.0 with concentrated ammonium hydroxide solution before use in enzyme preparations.

Preparations of reduced pyridine nucleotides. A modification of the dithionite reduction method of Conn et al. (57) was employed to reduce the DPN^+ analogues. 10 µmoles of oxidized pyridine nucleotide and 0.50 ml of 1.3% (w/v) $NaHCO_3$ were placed in a glass-stoppered test tube and the solution flushed with N_2.

6.0 mg of $Na_2S_2O_4$ were added and the solution incubated for 40 minutes at 25°. Unreacted hydrosulfite was removed by aeration for 4 minutes, and 15 ml of redistilled acetone at -15° were added. The precipitated nucleotide was collected by centrifugation and dried for 2 hrs at room temperature *in vacuo*. The nucleotide was then taken up in 2.0 ml of 0.01 M $NaHCO_3$ for assay and use. The extinction coefficients used to assay the reduced pyridine nucleotides were those given by Siegel *et al.* (58).

Assay of isocitric dehydrogenase activity.

A. DPN-linked isocitric dehydrogenase. The reaction mixture of Plaut and Sung (51) was used in the initial phases of this study. In a cuvette of 1.0 cm light path were placed 100 µmoles of cacodylate buffer, pH 6.5, 2 µmoles of $MnSO_4$, 1.0 µmole of DPN^+, 8.0 µmoles of *threo*-D_sL_s-isocitrate, enzyme and water in a final volume of 3.0 ml. The enzyme was added last, and the rate of density increase at 340 mµ was followed in a Zeiss PMQ II spectrophotometer. One unit of enzyme is defined as that amount which causes a change of 0.01 in optical density at 340 mµ per minute at 25° under these conditions.

An improved reaction mixture for assay was developed in the course of this study. The mixture consists of 100 µmoles of Tris acetate buffer, pH 7.2, 4.0 µmoles of $MnCl_2$, 1.0 µmole of DPN^+, 16.0 µmoles of *threo*-D_sL_s-isocitrate, enzyme and 2.0 µmole of ADP in a final volume of 3.0 ml. About 67% more apparent activity is obtained by the use of this improved reaction mixture. In the

present study, all enzyme activities are expressed in terms of the new assay procedure. The rationale for the modified reaction mixture is explained later in this chapter.

Specific activity is expressed as the number of units per mg of protein.

B. TPN-linked isocitric dehydrogenase. The reaction mixture is essentially that described by Plaut (20): In a cuvette of 1.0 cm path length are placed 100 μmoles of Tris acetate buffer, pH 7.2, 0.5 μmoles of TPN^+, 2.0 μmoles of $MnSO_4$, 8.0 μmoles of threo-D_sL_s-isocitrate, enzyme, and water in a final volume of 3.0 ml. Measurement of activity and the unit of activity are the same as described for the DPN-specific enzyme.

Preparation of DPN-linked isocitric dehydrogenase. Various methods for purification of enzyme from beef heart mitochondrial acetone powder were used in this study. These methods are detailed in Chapter III.

Preparation of TPN-linked isocitric dehydrogenase. Extensive purification of the TPN-specific isocitric dehydrogenase was not attempted; but small, partially purified preparations were needed in order to assay isocitrate, generate TPNH used in inhibition studies, and to perform other experiments described in subsequent chapters. Two simple methods proved practical. All operations were performed in the cold room (2-5°).

A. Carboxymethyl-cellulose chromatography (Modification of the method of Rose (59)).

1. Beef heart mitochondrial acetone powder, prepared according to Plaut and Sung (5), was extracted with 0.10 M potassium phosphate buffer in the ratio of 20 ml of buffer to 1 gm of acetone powder. Solid ammonium sulfate was added until the solution was 0.5 saturated, and the supernatant solution after centrifugation was retained. (In practice, the protein precipitating below 0.5 saturation was kept for purification of the DPN-linked isocitric dehydrogenase.)

2. A 50 ml aliquot of the above supernatant solution was treated with ammonium sulfate in the solid form, the pH being maintained at 7.0 to 7.4 by addition of solid Na_2CO_3. The protein precipitating between 0.6 and 0.8 saturation was collected by centrifugation, taken up in 2.0 ml of standard buffer, containing 5×10^{-4} M EDTA, pH 7.2) and dialyzed against 500 volumes of the same buffer for 2 hours.

3. The dialyzed enzyme was assayed for ammonia and diluted until the ammonium ion content was below 0.01 M. The enzyme was then applied to a CM-cellulose column, 1.2 x 6.5 cm, which had previously been equilibrated with the above standard buffer solution. Linear gradient elution was achieved by the use of a mixing chamber charged with 100 ml of standard buffer solution and a reservoir containing 100 ml of this buffer plus 0.2 M NaCl. A flow rate of about 1 ml per minute was maintained with a pressure of about 30 cm of water. Approximately 6 ml fractions were collected. The active fractions were kept separately and stored

at 2° after saturated ammonium sulfate solution was added to give a final concentration equal to 0.6 saturation.

The procedure is summarized in Table I.

TABLE I

PURIFICATION OF TPN-LINKED ISOCITRIC DEHYDROGENASE BY CM-CELLULOSE CHROMATOGRAPHY

Step	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg)
1. Supernatant solution (0.5 saturated $(NH_4)_2SO_4$)	50	200	2	100
2. $(NH_4)_2SO_4$ fractionation and dialysis	2.5	4080	10.2	400
3. CM-cellulose column				
Breakthrough fractions	11.6	438	1.10	400
Fraction 9	6.3	190	0.10	1900
Fraction 10	6.0	142	0.12	1200
Fraction 11	5.7	108	0.11	980
Fraction 12	5.7	80	0.11	740

B. Negative adsorption by DEAE-cellulose.

Protein which precipitated in ammonium sulfate solution between 0.6 and 0.8 saturation after being extracted from mitochondrial acetone powder as described above was dialyzed as in procedure A. About 30 mg of protein was placed on a 1 x 10 cm DEAE-cellulose column equilibrated against standard buffer solution. The enzyme had little affinity for the column and was eluted with the same buffer solution. Enzyme prepared in this was was virtually colorless, comes off the column in the first few fractions collected, and has a specific activity of about 7.5 units per mg. Somewhat less pure preparations have also been made by treating the dialyzed protein batch-wise with DEAE-cellu.ose and removing the resin on a Buchner funnel. For batch purification, 6 to 10 mg of DEAE-cellulose per mg of protein were used. Specific activities in the range of 650 units per mg were usually obtained.

Determination of initial reaction rate. Most of the routine assays for enzymic activity were sufficiently accurate if the optical density at 340 mμ was recorded every 30 seconds after initiating the reaction. For determination of Michaelis constants and for inhibition studies, however, care was taken to measure only the initial reaction rate. For these measurements, the thermostated cuvette chamber of the spectrophotometer was maintained at 25° by means of circulating water from a constant temperature bath (Haake Gebruder, Berlin, Germany). The reaction velocity was followed on a linear recorder (Photovolt Corp., N.Y.C.) and extrapo-

lation made to zero time.

Other determinations. Ammonia was determined by a modification of the Nessler method (60). Glutamate was determined by the method of Moore and Stein (61). Protein was determined by the method of Warburg and Christian (62). Fluorescence spectra were measured with a Farrand spectrophotofluorimeter fitted with a GE 100 watt AH-4 mercury lamp.

Chromatography of adenine nucleotides. In order to detect adenylate kinase activity in enzyme preparations, mixtures of enzyme and ADP were chromatographed on paper. Ascending chromatography was performed with Whatman No. 3MM paper in the system of Krebs and Hems (63), which consists of isobutyric acid:1 N ammonia: 0.1 M EDTA in the proportion 100:60:1.6. In the experiment involving ADP^{32}, phosphate was separated from adenine nucleotides by using the system of Hanes and Isherwood (64), which consists of isopropyl ether:90% (v/v) formic acid in the proportion 90:6.0.

II. RESULTS

Competitive Inhibition by DPNH, ATP, and ADPR. It was noted early in the course of these studies that the rate of DPNH formation in the DPN-linked isocitric dehydrogenase system diminished with time. The possibility that this effect could have been due to the enzymic reaction approaching a point of equilibrium could be ruled out immediately, since the DPN-linked isocitric dehydrogenase system seemed to be irreversible (5). Also, the analogous

reaction for the TPN-linked system had an equilibrium constant which would have been the same for the DPN-system had it been a reversible reaction, and the equilibrium constant is such that DPN^+ would be virtually completely reduced under the assay conditions (65). Product inhibition was considered, and it was found that neither α-ketoglutarate or CO_2 were inhibitory. However, added DPNH depressed the initial reaction rate, suggesting that the enzyme was inhibited by DPNH, a product of the reaction.

The inhibition by DPNH is illustrated in Figure 1A, which shows the decrease in reaction rate with time. If glutamic dehydrogenase, suspended in ammonium sulfate solution, is added to the reaction mixture, the absorbancy at 340 mμ due to DPNH disappears because α-ketoglutarate, DPNH, and ammonia react to yield glutamate and DPN^+. This is shown by the rapid fall in optical density at 340 mμ in Fig. 1A when glutamic dehydrogenase (GDH) is added at the time indicated by the arrow. One would expect that the rate of isocitrate utilization would then be linear, since there would be no DPNH accumulation, and the following dismutation could, therefore, occur:

$$\underline{\text{threo-D}_s}\text{-isocitrate} + DPN^+ \longrightarrow \alpha\text{-ketoglutarate} + CO_2 + DPNH + H^+$$

$$\alpha\text{-ketoglutarate} + DPNH + H^+ + NH_3 \longrightarrow \underline{\text{L}}\text{-glutamate} + DPN^+$$

$$\text{Sum: } \underline{\text{threo-D}_s}\text{-isocitrate} + NH_3 \longrightarrow \underline{\text{L}}\text{-glutamate} + CO_2$$

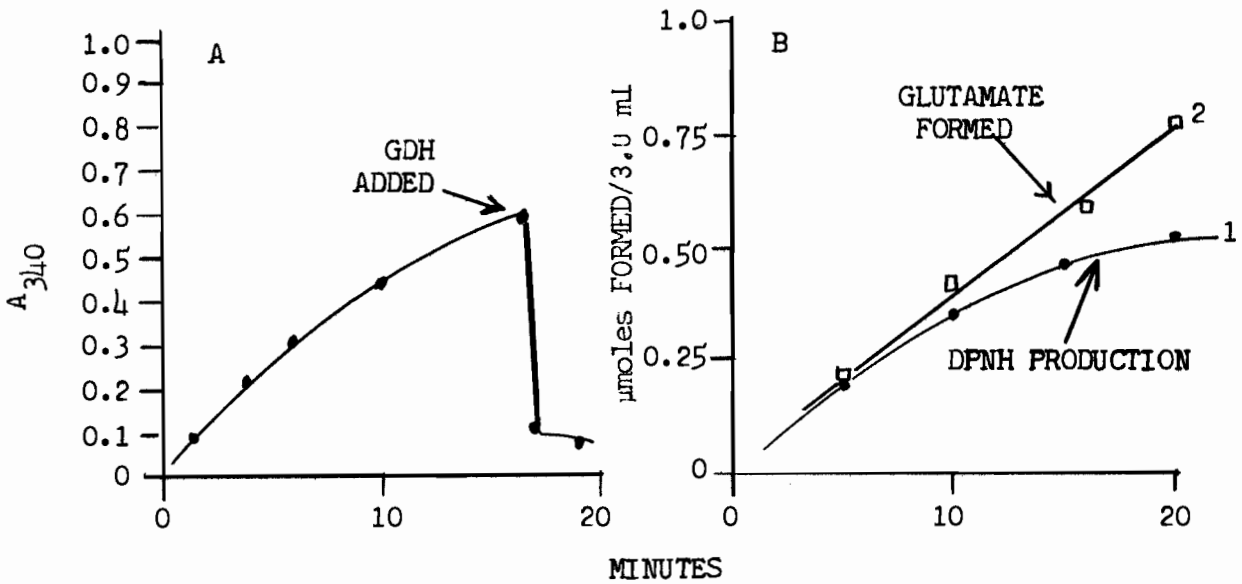

Fig. 1. Effect of DPNH removal on the rate of isocitrate oxidation.

A. The reaction mixture contained 3.3×10^{-2} M cacodylate buffer, pH 6.5; 6.7×10^{-4} M $MnCl_2$; 2.7×10^{-3} M threo-D_sL_s-isocitrate; 10^{-2} M NH_4Cl; DPN-linked isocitrate dehydrogenase, and water in a volume of 3.0 ml. At the time indicated, 0.2 mg of crystalline glutamate dehydrogenase (GDH) was added.

B. Curve 1 shows the formation of DPNH in the presence of DPN-specific isocitrate dehydrogenase. The composition of the medium was similar to that in Part A. Curve 2, the conditions were similar to those prevailing for curve 1 except that 0.2 mg of glutamate dehydrogenase was present before initiation of the reaction with DPN-specific isocitrate dehydrogenase.

In order to confirm that the rate of isocitrate oxidation under conditions where this dismutation reaction can occur, however, optical density change at 340 mμ could be used since no reduced pyridine nucleotide accumulates. However, isocitrate utilization could be followed by stopping the reaction at various times and assaying glutamate by the ninhydrin method. Such an experiment is shown in Fig. 1B, and shows that removal of DPNH did indeed allow isocitrate utilization to proceed at a constant rate.

Further investigation of the DPNH effect revealed that DPN^+ could reverse the inhibition by DPNH. A series of experiments done at pH 6.5 in cacodylate buffer indicated that the DPNH inhibition was competitive with DPN^+, since Lineweaver-Burk (66) plots of 1/v vs. $1/[DPN^+]$ at various DPNH concentrations met at common point on the ordinate. The same results were obtained when these experiments were repeated at pH 7.2 in the presence of ADP. Fig. 2B shows that under these conditions, the inhibition by DPNH is still competitive with DPN^+. K_m for DPN^+ and K_I for DPNH were calculated by the method of Dixon (67) to be 7.8×10^{-5} M, and 3.9×10^{-5} M, respectively, suggesting that the enzyme has greater affinity for DPNH than for DPN^+.

It was of interest to compare the affinities of the two isocitric dehydrogenases for their respective pyridine nucleotide coenzymes. However, TPN-linked isocitric dehydrogenase has such a high affinity for both TPN^+ and TPNH that the Michaelis constants for these nucleotides have never been determined spectrophoto-

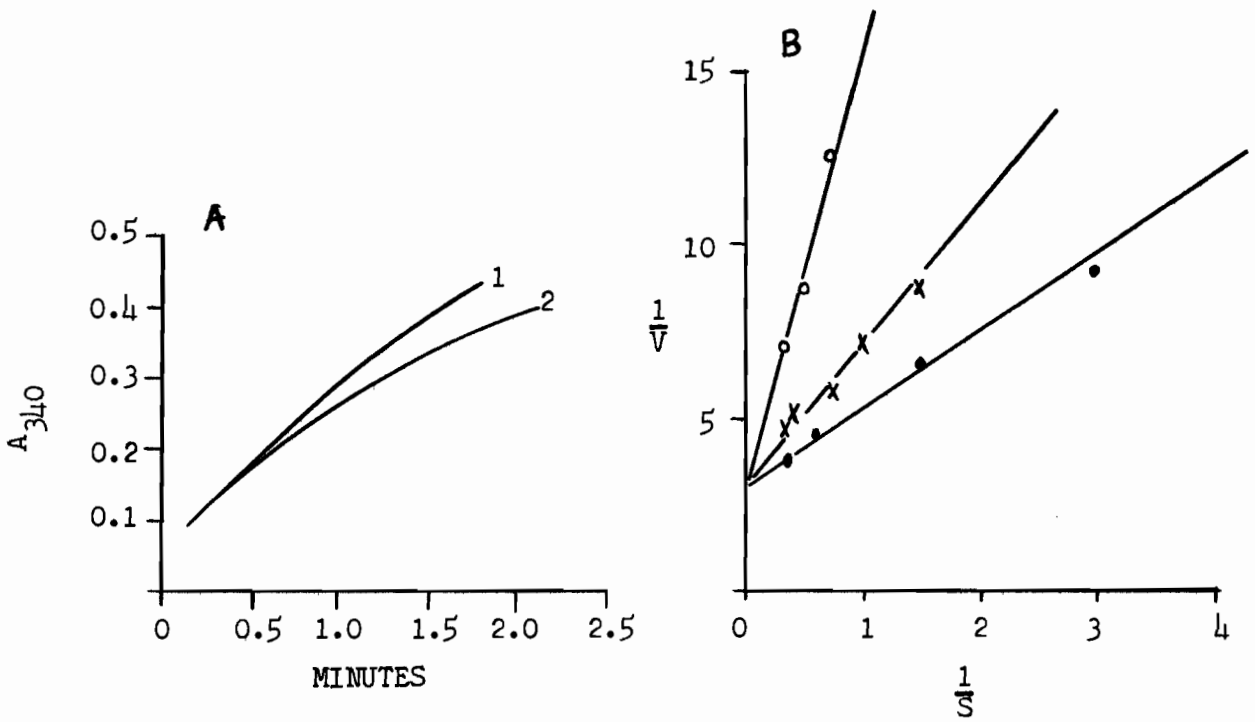

Fig. 2. Inhibition by DPNH and TPNH.

A. Continuous recordings showing the optical density at 340 mμ as a function of time. The cuvettes contained the usual assay system in a volume of 3.0 ml, including, Curve 1, 3.3×10^{-4} M DPN^+; Curve 2, 3.3×10^{-4} M DPN^+ and 3.3×10^{-4} M TPNH. 17 μg of protein per cuvette (specific activity, 3200).

B. Plot of the reciprocal of velocity against the reciprocal of DPN^+ concentration. The usual assay system was used for each reaction except that the DPN^+ content was varied and inhibitor was added in the experiments as indicated below. •———• , no reduced pyridine nucleotides were present initially; x———x, 3.3×10^{-5} M DPNH present initially; o———o, 3.3×10^{-5} M DPNH and 3.3×10^{-5} TPNH were present initially. V, velocity expressed as change in optical density at 340 mμ per minute. S, concentration of DPN^+, M x 10^3. Enzyme, same as in Part A.

metrically, as pointed out by Ochoa (65). However, it has been possible, in the present studies to determine K_m for TPN^+ fluorimetrically. The double reciprocal plot for the data obtained is shown in Fig. 3. K_m for TPN^+ was calculated to be 3.0×10^{-6} M. The enzyme preparation used was quite impure, having a specific activity of about 100. The preparation consisted merely of the supernatant fraction of the crude extract of mitochondrial acetone powder following ammonium sulfate precipitation of protein at 0.5 saturation. Langan (68) subsequently reported that TPN-linked isocitric dehydrogenase of pig heart combined with TPNH to form a distinctive fluorescent complex. He calculated the dissociation constant to be about 10^{-8} M. TPN^+ competitively displaced TPNH, but the affinity of the enzyme for TPN^+ appeared to be only 1/200 that for TPNH. Thus, the dissociation constant for TPN^+ would be 2×10^{-6} M, a value rather close to the K_m found fluorimetrically in the present kinetic studies. Both isocitric dehydrogenases, therefore, have a greater affinity for the reduced forms of their respective coenzymes.

The inhibition of DPN-linked isocitric dehydrogenase by ATP was also noted to be reversed by DPN^+, and experiments at both pH 6.5 and pH 7.2 indicated that the inhibition was of the competitive type (Fig. 4A). Neither ATP nor DPNH was competitive with isocitrate, however,

ADPR was also tested and found to give Lineweaver-Burk curves showing inhibition competitive with DPN^+. Calculation of the

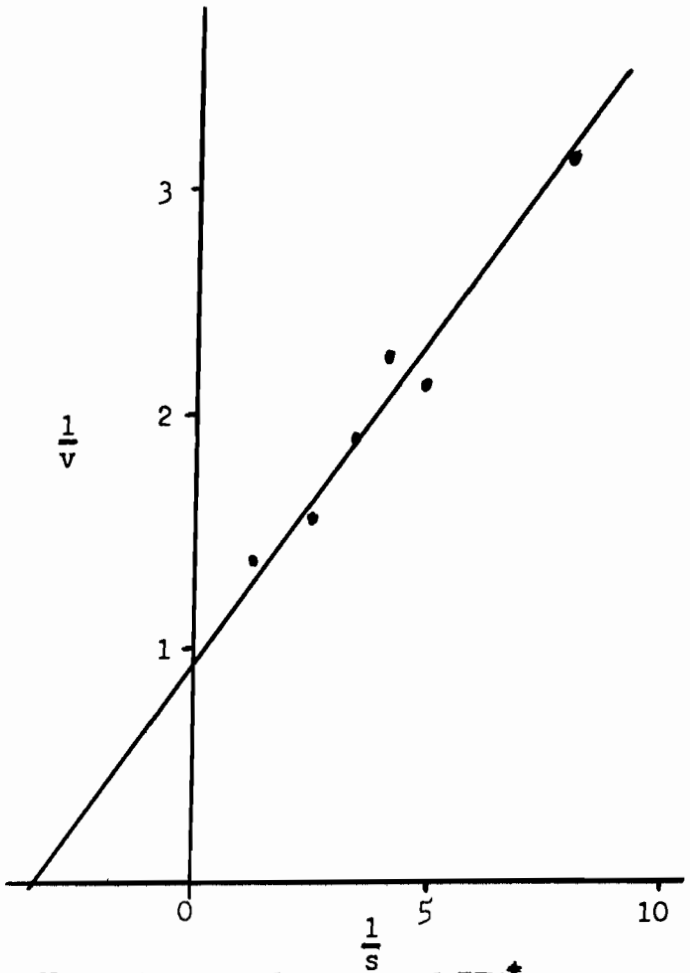

Fig. 3. Velocity as a function of TPN^+ concentration in the TPN-linked isocitrate dehydrogenase system.

The reciprocal of velocity (V, the change in fluorescence intensity at 460 mμ per minute, expressed as mm of recorder deflection x 10^{-2}) is plotted against the reciprocal of TPN^+ concentration (S, the concentration of TPN^+, M x 10^5). The measurements were done at room temperature, 23-25°. The reaction mixtures contained 100 μmoles of threo-D_sL_s-isocitrate, water, enzyme, and TPN^+ in a final volume of 3.0 ml. The TPN^+ concentration was varied in the range of 1-4 x 10^{-6} M. 0.1 mg of protein was used per cuvette (specific activity, 100).

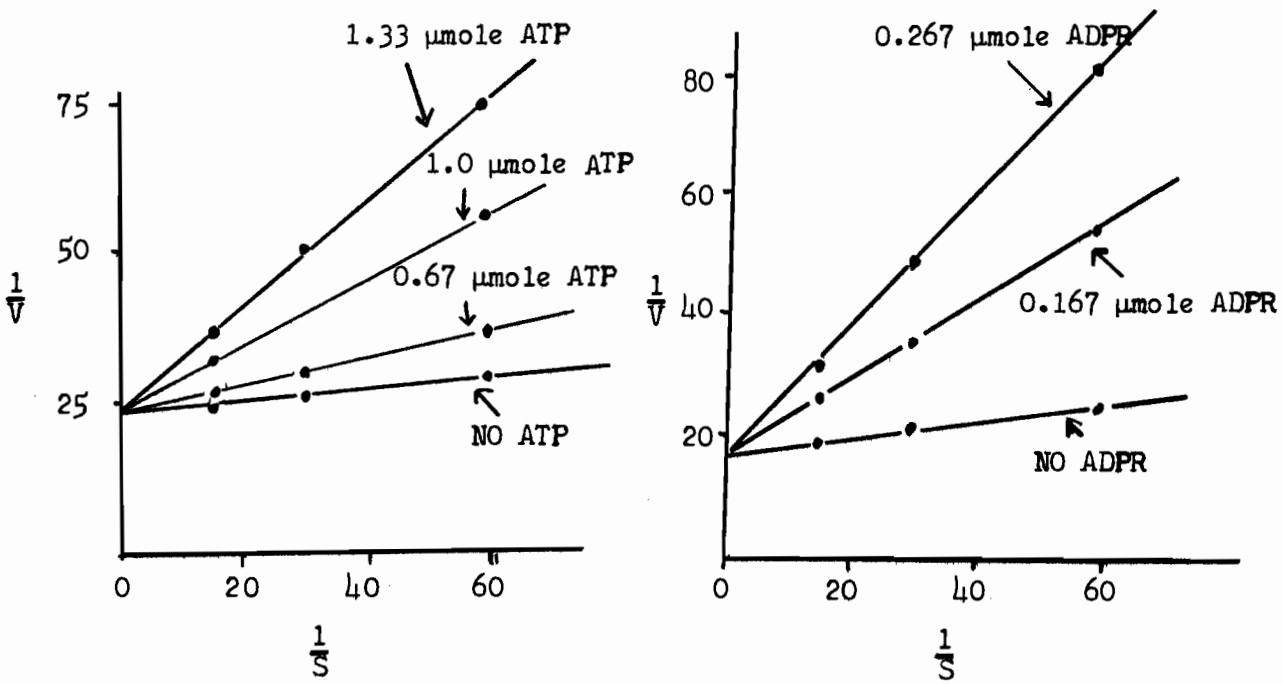

Fig. 4. Inhibition by ATP and ADPR.

Plot of the reciprocal of velocity against the reciprocal of DPN^+ concentration. The usual assay system was employed except that the DPN^+ content was varied and inhibitor was added in the amounts indicated. Enzyme, 15 µg per cuvette (specific activity, 400).. V is the change in optical density at 340 mµ per minute, and S is the concentration of DPN^+ in M x 10^2.

inhibition constants for ATP and ADPR yielded values of 1.5×10^{-4} M and 6.1×10^{-5} M, respectively.

Inhibition by Other Nucleotides. A number of other nucleotides were inhibitory (Table II). However, rather large amounts of these

TABLE II

INHIBITION BY VARIOUS NUCLEOTIDES

Nucleotide	Concentration for approximately 50% inhibition
	M
IDP	3.0×10^{-3}
CDP	3.0×10^{-3}
ATP	3.5×10^{-4}
ITP	1.7×10^{-3}
UTP	1.7×10^{-3}
2'-AMP	1.5×10^{-2}
3'-AMP	1.5×10^{-2}
5'-AMP	1.5×10^{-2}
ADPR	1.4×10^{-4}

The conditions of incubation were as described under "Assay of enzymic activity" except that ADP was omitted. The reactions were initiated with enzyme (specific activity, 400).

nucleotides were required for inhibition in contrast to the amounts needed with ATP and ADPR. The inhibition by these nucleotides, therefore, may be due at least partly to chelation of Mn^{++}. Mn^{++}

concentration in these experiments was 0.0013 M. This may explain the 50% inhibition of DPN-linked isocitric dehydrogenase by 0.015 M 2'-AMP, a nucleotide which has been postulated to inhibit TPN-linked enzymes specifically and to have little effect on DPN-specific enzymes including DPN-linked isocitric dehydrogenase (28).

Stimulation of activity by ADP. Since DPNH, ATP, and ADPR all were competitive inhibitors having an adenosine pyrophosphoryl moiety as part of their structures, ADP was expected also to inhibit. However, when the enzyme was assayed at pH 6.5 in the "old assay" (see Materials and Methods) in the presence of ADP, approximately 20% more activity was found than in the control. The per cent stimulation by ADP, however, varied with the amount of isocitrate present. It was also found that the optimal amount of ADP seemed to be about 10^{-3} M although stimulation could be detected at concentrations as low as 6.7×10^{-5} M. It is apparent from the two curves shown in Fig. 5 that V'/Vc, the ratio of the rate in the presence of ADP (V') to the rate without ADP (Vc), is greater at the lower isocitrate concentration. It seemed possible that such an effect could be due to an increased affinity of the enzyme for isocitrate in the presence of ADP. Therefore, the effect of varying the isocitrate concentration on the reaction rate was investigated, both in the presence and in the absence of ADP. The results of kinetic studies done at pH 7.2 and 6.5 are expressed as Lineweaver-Burk plots in Fig. 6A and 6B. From each line in Fig. 6A and 6B, a Michaelis constant can be calculated by the method of Dixon (67).

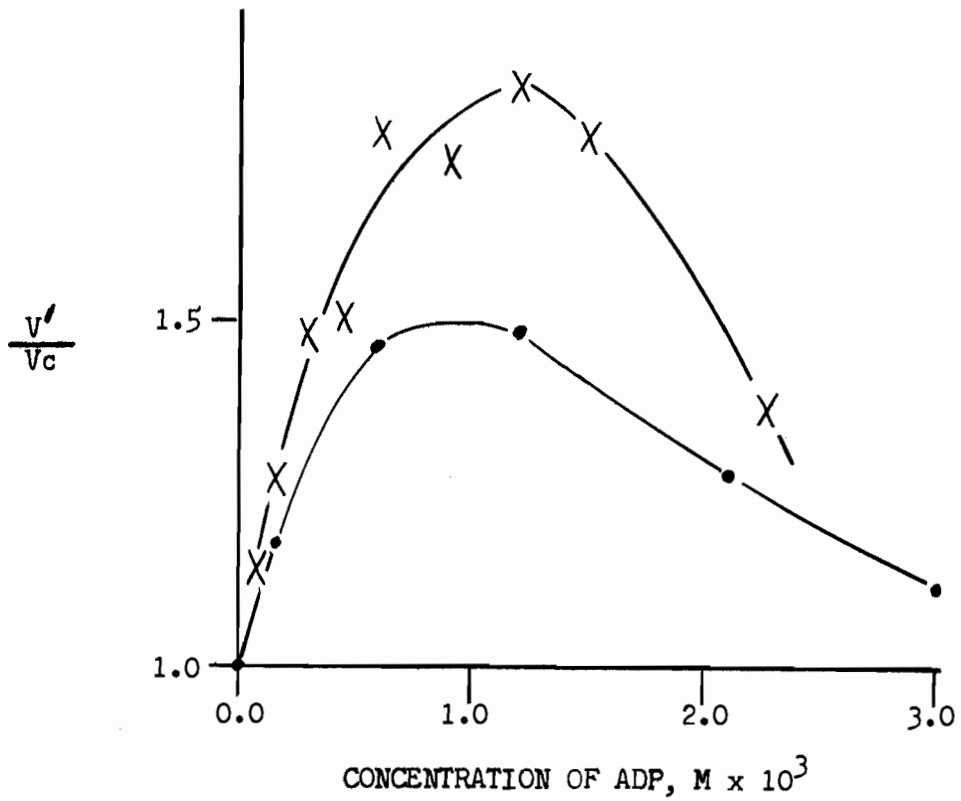

Fig. 5. The effect of ADP on initial reaction rates as a function of ADP concentration

All measurements were taken at 25°. Each cuvette contained in a final volume of 3.0 ml: 100 μmoles of Tris acetate buffer, pH 7.2; 4.0 μmoles of $MnCl_2$; 1.0 μmoles of DPN^+, and 10 μg. of enzyme (specific activity, 1001 units per mg). The concentrations of ADP and isocitrate were varied. x——x, 6.7×10^{-4} M isocitrate; •——• , 2.7×10^{-3} M isocitrate. V' is the rate in the presence of ADP and Vc is the rate in the absence of ADP. The reactions were initiated by the addition of enzyme.

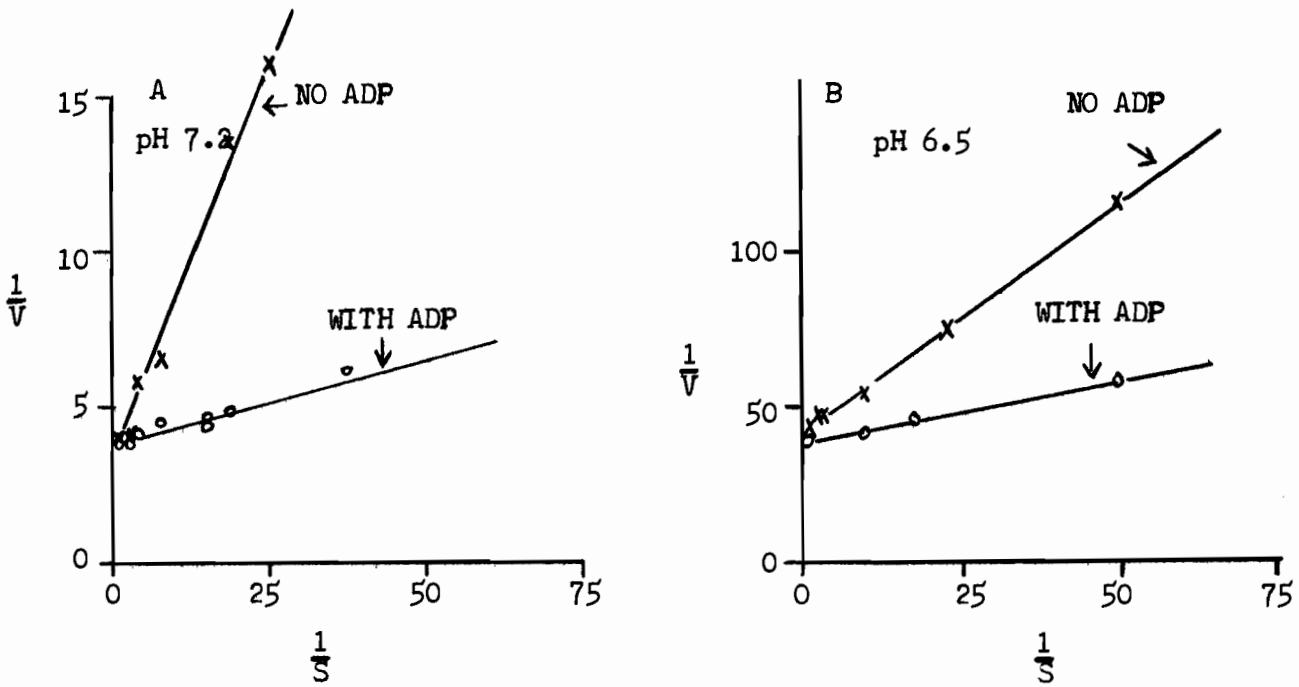

Fig. 6. Velocity as a function of isocitrate concentration in the presence and absence of ADP.

The reciprocal of velocity (V, change in optical density at 340 mμ per minute) is plotted against the reciprocal of isocitrate concentration (S, concentration of threo-D_s-isocitrate, M x 10^2) for experiments done at pH 7.2 (A) and at pH 6.5 (B). The conditions of incubation were the same as described under "Materials and Methods", except that the concentration of isocitrate was varied and 6.7×10^{-4} M ADP was either present (o——o) or absent (x——x) from the reaction mixtures. In addition, in the experiments of Fig. 6B, the usual Tris buffer was replaced by cacodylate buffer, pH 6.5. In the experiments of Fig. 6A, 25 μg of protein (specific activity, 1001) were used per cuvette; about 22 μg of protein (specific activity, 200) per cuvette were present in the experiment represented by Fig. 6B.

The results of such calculations are given in Table III. It is clear that ADP diminishes the K_m for isocitrate at both pH 7.2 and pH 6.5, the effect being greater at the former pH. Thus, the K_m

TABLE III

K_m FOR ISOCITRATE WITH AND WITHOUT ADP

Conditions	K_m
pH 6.5, no ADP	3.6×10^{-4} M
pH 6.5, 6.7×10^{-4} M ADP	1.0×10^{-4} M
pH 7.2, no ADP	1.5×10^{-3} M*
pH 7.2, 6.7×10^{-4} M ADP	1.4×10^{-4} M*

These values are calculated from the data of Fig. 2.

*The present K_m values were obtained with a more highly purified preparation of enzyme than used in an earlier study (69) which indicated that ADP reduced the K_m at this pH by at least 5-fold.

for isocitrate is reduced some ten-fold at pH 7.2 from 1.4×10^{-3} M in the absence of ADP, to 1.3×10^{-4} M in the presence of ADP. The presence of ADP at pH 6.5 produces only a 3.6-fold decrease in K_m for isocitrate. Because the lines in Fig. 6A and 6B converge at the ordinates, V_{max} is not changed by ADP. It is clear, then that the effect of ADP is to increase the apparent affinity of the enzyme for substrate.

It should also be noted that Fig. 6 illustrates the fact that

the stimulatory effect of ADP becomes more dramatically evident when the isocitrate concentration is low. Thus, at pH 7.2 it can be shown that when the isocitrate concentration is 1×10^{-3} M, the rate in the presence of ADP is 1.5 times greater than in its absence; but at a lower concentration of isocitrate, 1×10^{-4} M, the stimulated rate is about 70 times greater than the control. Thus, the enzyme is virtually dependent on ADP for activity if substrate concentration is relatively low.

ADP also lowers K_m for divalent metal ions. The data of Plaut and Sung (5) suggested that the K_m for Mn^{++} was in the region of 1×10^{-4} M at pH 6.5 in the absence of ADP. Since the assay conditions were changed in order to have the reaction run at pH 7.2 with 6.7×10^{-4} M ADP present, the K_m for Mn^{++} was reinvestigated in order to ascertain the optimal concentration of Mn^{++} to be employed, as well as to see if ADP affected the apparent affinity of the enzyme for Mn^{++}. The data of experiments relevant to these problems are plotted as reciprocal velocity vs. the reciprocal of Mn^{++} concentration both in the presence and absence of ADP at pH 7.2 in Fig. 7. In these experiments, saturating amounts of isocitrate and DPN^+ were present. It was shown that 6.7×10^{-4} M ADP decreases K_m for Mn^{++} from 2.1×10^{-4} M to 2.7×10^{-5} M: an 8-fold diminution. V_{max} is the same with and without ADP.

Plaut and Sung (5) found that Mg^{++} could satisfy the metal requirement of DPN-linked isocitric dehydrogenase. In the experiments of Fig. 7B, it was found that ADP affected the Michaelis

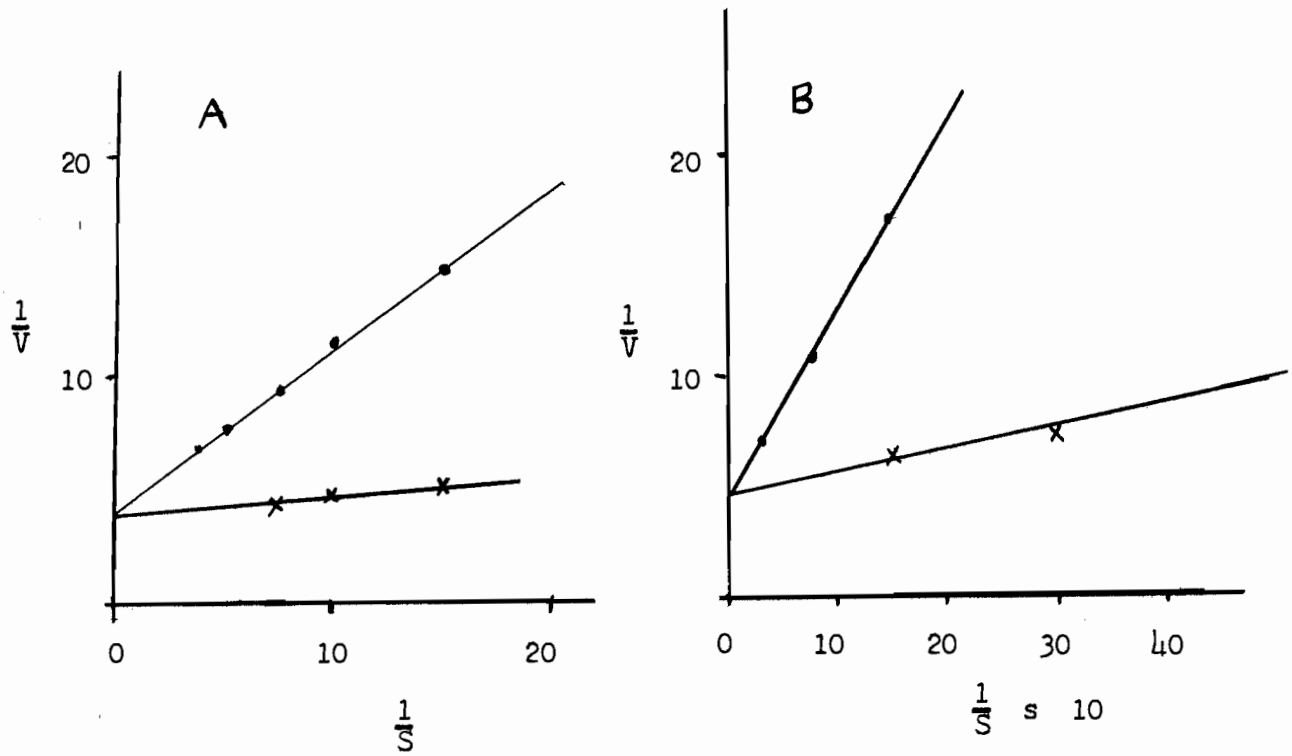

Fig. 7. Velocity as a function of Mn^{++} and Mg^{++} concentration in the presence and absence of ADP.

The reciprocal of velocity (V, change in optical density at 340 mμ per minute) is plotted against the reciprocal of metal ion concentration (S, the concentration of metal ion, M x 10^{2}) for experiments done for Mn^{++} (A) and Mg^{++} (B). The conditions for the reactions are the same as in Fig. 6, except that isocitrate concentration was kept constant at 2.7 x 10^{-3} M. In each experiment, the cuvette contained 10 μg of protein, specific activity 3000 units per mg. •——• , no ADP; x——x, 6.7 x 10^{-4} M ADP present.

constant for Mg^{++} at pH 7.2 just as it had in the case of Mn^{++}. Without ADP, K_m for Mg^{++} was found to be 1.8×10^{-3} M, while in the presence of 6.7×10^{-4} M ADP, K_m for Mg^{++} decreased to 1.8×10^{-4} M. V_{max} is again the same with and without ADP. It is interesting that while the Michaelis constants for Mg^{++} are much higher than those for Mn^{++}, the V_{max} for Mg^{++} is only 20% lower than that for Mn^{++}.

The effect of ADP on the apparent affinity of the enzyme for both Mn^{++} and Mg^{++} is of the same order of magnitude as the effect on the K_m for isocitrate. Chelation of metal by ADP cannot explain the decrease in K_m for the metal ion, since such binding would give an apparent <u>increase</u> in K_m by removal of ions available for binding to the enzyme. The fact that ADP influences the binding of both isocitrate and metal ion, but not that of DPN$^+$, suggests that metal ions are involved in the substrate binding site but not in the coenzyme site.

A further effect of ADP is to shift the pH optimum of the DPN-specific isocitric dehydrogenase system to pH 7.2. It was reported by Plaut and Sung (5) that maximal activity occurred at pH 6.5 in the presence of cacodylate buffer and 1.3×10^{-3} M <u>threo-D$_s$</u>-isocitrate. Fig. 8 shows the reaction rate as a function of the pH for high and low concentrations of isocitrate as determined in imidazole chloride and Tris acetate buffers. The results of Plaut and Sung (5) were essentially confirmed because it can be seen that with an isocitrate concentration of 1.3×10^{-3} M, the pH optimum appears to be 6.7. (It has been noted that activity in

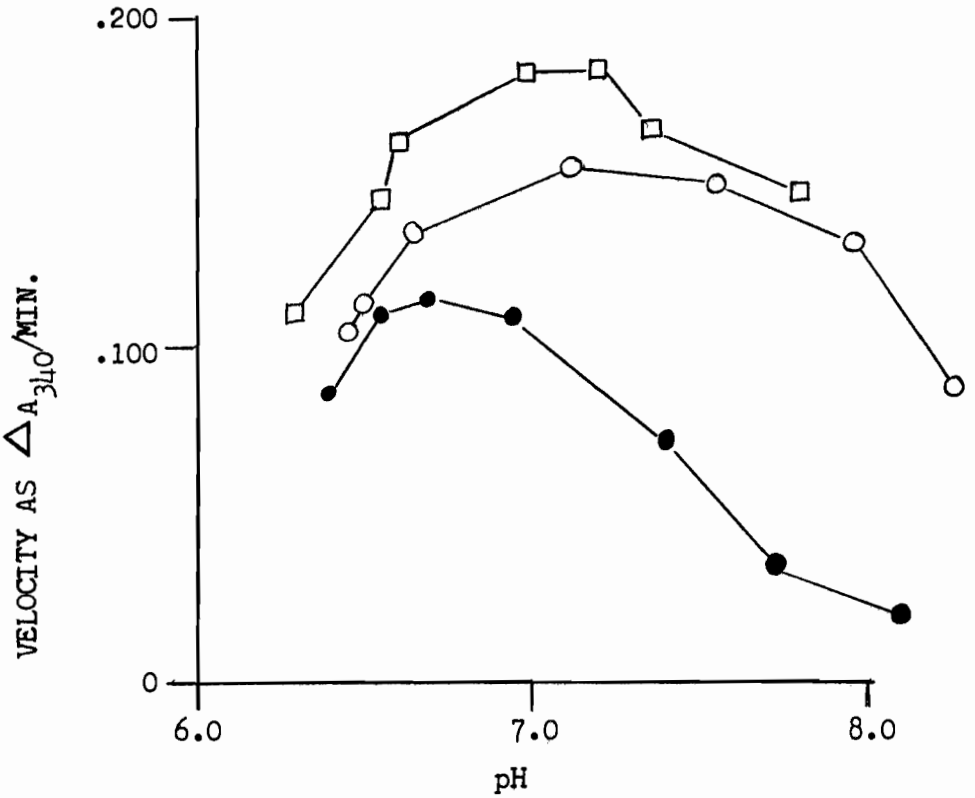

Fig. 8. Effect of isocitrate and ADP on pH optimum.

All reactions were run at 25° and initiated by addition of enzyme. Each reaction mixture of 3.0 ml contained 100 μmoles of imidazole chloride buffer (below pH 7.0) or Tris acetate buffer (pH 7.0 or above), 4.0 μmoles of $MnCl_2$, 1.0 μmole of DPN^+, 25 μgrams of protein (specific activity, 400). Isocitrate and ADP were varied as follows: □-----□ , 1.3×10^{-3} M threo-D_s-isocitrate and 6.67×10^{-4} M ADP; o-----o, 1.3×10^{-2} M threo-D_s isocitrate and no ADP; •———• , 1.3×10^{-3} M threo-D_s-isocitrate and no ADP.

cacodylate buffer is slightly higher than in imidazole buffer at any given pH.) When 6.7×10^{-4} M ADP is present, however, the pH optimum is seen to shift to 7.2. An optimum at pH 7.2 is also obtained if isocitrate concentration is increased to 1.3×10^{-2} M in the absence of ADP (Fig. 8, middle curve). Fig. 8 indicates that at the lower isocitrate concentration in the absence of ADP, the reaction rate at pH 7.2 is much slower than at pH 6.5-6.7. This observation can be explained at least partially by the fact that K_m for isocitrate in the absence of ADP is about 4 times higher at pH 7.2 than at pH 6.5 (Table III). The apparent affinity of the enzyme for substrate is actually greater at low pH, but the velocity falls rapidly on the acid side of pH 6.5 probably due to protonation of a carboxylate group of isocitrate (the third ionization constant of citric acid, pK_a, has been found to be 5.7 at an ionic strength of 0.12 (70)).

Two possible explanations for the ADP stimulation were tested: 1. That ADP is a participant in an overall reaction in which the nucleotide is consumed to yield orthophosphate and 5'-AMP or an adenylic acid derivative. 2. That ADP reacts with the enzyme leading to the formation of an activated enzyme complex.

The possibility that ADP participates in stoichiometric amounts in the DPN-linked isocitric dehydrogenase reaction was tested by using ADP labeled in the terminal phosphate group with P^{32}. In a final volume of 0.3 ml were placed 10 μmoles of Tris acetate buffer, pH 7.2; 0.4 μmoles of $MnSO_4$; 1.6 μmoles of threo-$D_s L_s$-

isocitrate; 0.1 μmole of DPN^+; 0.2 μmole of ADP^{32}; and 3 units of enzyme, specific activity 725 (4.1 μg total). After 35 minutes at 23°, the reaction had gone almost to completion and the mixture was deproteinized with 10% trichloroacetic acid, and an aliquot of the supernatant fluid was chromatographed on paper in the isopropyl ether: formic acid system of Hanes and Isherwood (64) and in the isobutyric acid:ammonia:EDTA system (63). Radioactivity was located by radioautography and found in the area corresponding to ADP, except for a small amount in ATP; no radioactivity was found in the area corresponding to orthophosphate. Since the ATP spot also occurred to the same extent in a control reaction mixture not containing isocitrate, adenylate kinase contamination was probably present. Direct tests on similar preparations of enzyme showed the presence of adenylate kinase. ADP, therefore, seems to stimulate the enzyme in catalytic rather than in stoichiometric amounts.

If the addition of ADP led to the formation of an activated enzyme complex, it seemed possible that a lag period might occur between the addition of ADP and the attainment of full stimulation. Kearney (71) found that the stimulation of succinic dehydrogenase by malonate, succinate, or phosphate was a relatively slow process involving large energy changes. To test this possibility, the experiment depicted in Fig. 9 was performed. When a reaction was started in the absence of ADP, and the nucleotide added at a later time, the stimulation of reaction rate seemed to

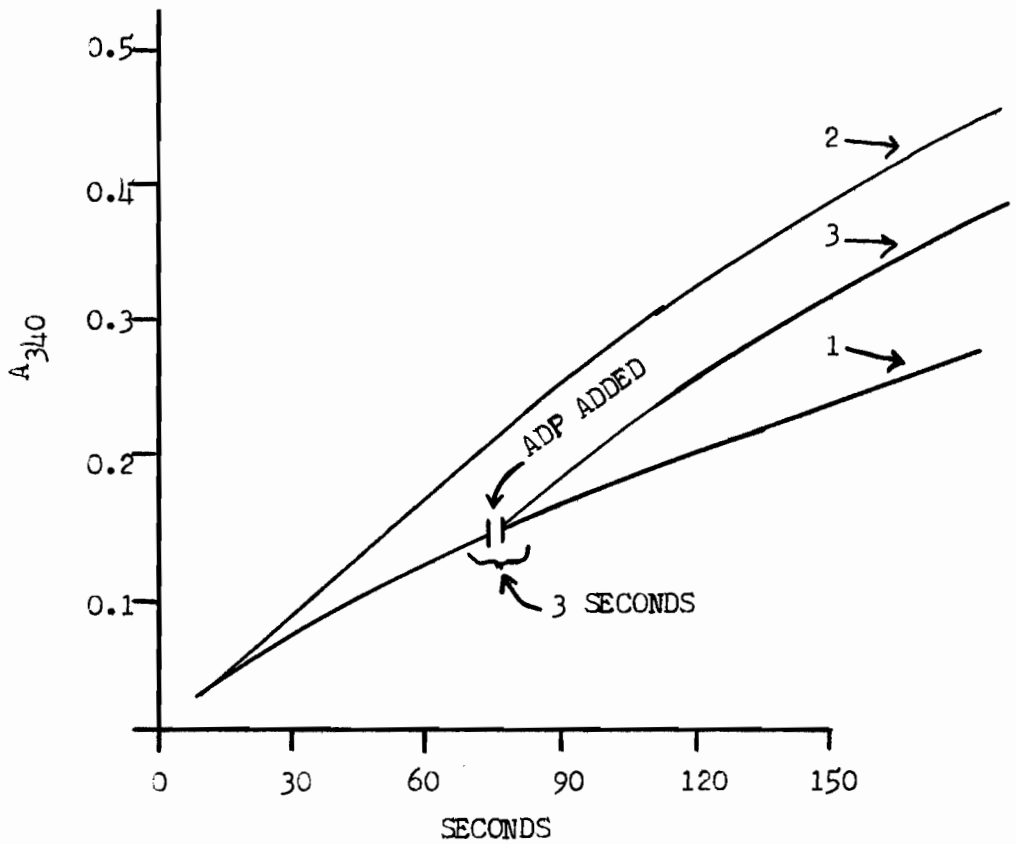

Fig. 9. The effect of the delayed addition of ADP on velocity.

Each cuvette contained in a volume of 3.0 ml: 100 μmoles of Tris acetate buffer, pH 7.2; 16.0 μmoles of threo-D_sL_s-isocitrate; 4.0 μmoles of $MnSO_4$ and 1.0 μmole of DPN^+. The reactions were initiated by adding 6.2 μg of enzyme protein (specific activity, 2670) and incubated at 25°. The progress of the reaction was followed at 340 mμ in a Cary recording spectrophotometer (Model 14). Curve 1, reaction in the absence of ADP. Curve 2, all components of the reaction mixture including 2.3×10^{-4} M ADP present initially. Curve 3, ADP was absent initially. At the time indicated by the arrow, the cuvette chamber was opened and 0.69 μmole of ADP was mixed with the incubation solution by means of a polyethylene spatula. The cuvette compartment was closed and the recording of the progress of the reaction resumed.

occur immediately and to the same degree as when ADP was present initially. No delay in the onset of the ADP, thus, was observed, and the techniques used in this experiment would have detected any lag period of three seconds or more.

That ADP seems to affect the binding of isocitrate to the enzyme has been indicated above. However, ADP does not seem to affect the site of DPN^+ binding, because the K_m of DPN^+ was about 10^{-4} M both with and without ADP at pH 7.2. Furthermore, a number of experiments indicated about the same per cent stimulation of reaction rate by ADP in the presence of either DPN^+ or acetylpyridine-DPN^+. Additional evidence that ADP does not affect the coenzyme site is the observation that DPNH inhibition, which is competitive with DPN^+, is not reversed by ADP.

The activation by ADP seems to be specific, since a large number of related compounds had no such effect when tested in the concentration range of 1×10^{-3} M to 1×10^{-4} M. Thus, no significant stimulation was exerted by the following: adenine, adenosine, 2'-AMP, 3'-AMP, 5'-AMP, 3',5'-cyclic-AMP, dGMP, dAMP, UMP, ADPR, IDP, GDP, UDP, ITP, dADP seemed possibly 10% more stimulatory than ADP at a concentration of 6.7×10^{-4} M. The fact that only ADP and dADP stimulate the enzyme suggests that the activation is an intrinsic property of DPN-linked isocitric dehydrogenase and makes a non-specific activation, such as chelation of a metal impurity, extremely unlikely as the basis of ADP stimulation. The enzyme does not seem to contain tightly bound ADP, because treatment of

enzyme with Norit A (about 1 mg per mg of protein) under conditions of low ionic strength failed to give any change in the per cent stimulation by ADP added subsequently. Moreover, the degree of activation by ADP seemed to stay constant in each step of all purification procedures. The binding of ADP to enzyme thus seems rather weak. This finding is also suggested by the fact that half maximal stimulation, as shown in Fig. 5, appears to occur at around 4 to 5 x 10^{-4} M ADP, a relatively high value in comparison to the value found for the yeast DPN-linked isocitric dehydrogenase, for which half-maximal activation by 5'-AMP occurred at a concentration of 9 x 10^{-6} M nucleotide (4).

Orthophosphate was tested for stimulation because Ramakrishnan and Martin (30) had reported stimulation of the DPN-linked isocitric dehydrogenase of A. niger by phosphate. The beef heart enzyme was dialyzed free of phosphate against Tris acetate buffer and was found still to be active, although the prolonged dialysis caused some loss of activity. Addition of phosphate did not stimulate the enzyme either in the presence or absence of ADP.

Effect of TPNH. It was noted that the amount of isocitrate oxidized in the presence of TPNH in a given period of time was less than obtained in the absence of TPNH. When TPNH was tested for inhibition of the initial reaction rate, however, no consistent results were obtained. Certain commercial preparations of TPNH seemed to yield inhibition of the initial rate[3], but purified preparations

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[3]Samples from Biochimica-Boehringer, distributed by California Corporation for Biochemical Research.

uniformly gave uninhibited initial rates. However, the rate of decrease in reaction velocity with time was increased over controls. Typical curves representing reactions with and without TPNH are shown in Fig. 2A. TPNH apparently potentiates the inhibitory effect of DPNH, so that the usual decrease in velocity with time due to product inhibition by DPNH (Fig. 2A, curve 1) becomes more marked in the presence of TPNH (Fig. 2A, curve 2). The magnitude of this effect of TPNH was examined by calculating the K_I for DPNH in the presence and absence of TPNH from Lineweaver-Burk plots such as shown in Fig. 2B. K_I for DPNH was calculated to be 3.9×10^{-5} M. In the presence of 3.3×10^{-5} M TPNH and an equimolar amount of DPNH, the potentiated inhibition resulted in an apparent inhibition constant, K_I, of 0.76×10^{-5} M. It can be calculated that, under standard assay conditions, 0.1 μmole of DPNH, in the presence of 0.1 μmole of TPNH, exerts the same amount of inhibition as about 0.5 μmole of DPNH alone.

Since impurities, such as DPNH, might have been present in commercial TPNH samples, TPNH was prepared for inhibition studies in two ways. In the first method, TPNH was generated enzymically in the presence of the DPN-linked isocitrate dehydrogenase reaction system by means of glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and TPN*. Isocitrate oxidation then was initiated by addition of DPN-linked isocitrate dehydrogenase. TPNH was also prepared by reduction of TPN in the TPN-linked isocitrate dehydrogenase system and purified on DEAE-cellulose columns (72) as

described in Chapter IV. The purified TPNH had a ratio of optical densities at 260 mμ and 340 mμ of 2.4 at pH 7.2. TPNH prepared by either method gave the same results, as did Sigma Chemical Co. preparations.

The possibility that the effect of TPNH could have been an artefact due to contaminating enzymes was considered. Thus, TPN-linked isocitrate dehydrogenase or glutamate dehydrogenase could decrease the rate of increase in optical density at 340 mμ with time by reoxidizing TPNH with α-ketoglutarate formed in the DPN-linked isocitrate dehydrogenase reaction. This possibility seemed remote since the enzyme used for the study seemed to be at least 60% pure by ultracentrifugation. In fact, neither enzyme could be demonstrated in the DPN-specific isocitric dehydrogenase preparation used. Moreover, TPNH potentiated the inhibition of the initial reaction rate when <u>both</u> nucleotides were present initially (Fig. 2B). It was also conceivable that pyridine nucleotide transhydrogenase could lead to an apparent augmentation in DPNH inhibition by producing more DPNH from DPN^+ and TPNH. However, transhydrogenase did not seem to be present, since DPN^+, TPNH, and enzyme did not result in the formation of TPN^+, as tested for by the glucose 6-phosphate dehydrogenase system. Also, the TPNH effect could not be explained by transhydrogenase since the amount of inhibition obtained in the presence of equimolar amounts of TPNH andDPNH was consistently greater than that obtainable if all the TPNH had been converted to DPNH (i.e., doubling the DPNH

concentration in the absence of TPNH).

Reactivity with DPN analogues. Table IV shows the reactivity of the DPN-linked isocitrate dehydrogenase system with a number of DPN^+ analogues. The 3-acetylpyridine analogue was the only one which oxidized substrate at a significant rate (50% that of DPN^+). The enzyme does not seem to interact with the deamino analogue of DPN^+ since there was no reduction of this compound, and it did not inhibit the rate of the reaction with DPN^+. An intact adenosine 5'-pyrophosphoryl moiety may be required for binding of the nucleotides to the coenzyme site since ATP, ADPR, and DPNH inhibit competitively with DPN^+ whereas deamino-DPNH and TPNH do not.

The studies on the ability of various DPN^+ analogues to replace DPN^+ should be taken only as a qualitative survey for the following reason. The analogues were tested only at a concentration of 3.3×10^{-4} M, the same concentration which is used for DPN^+ in the standard assay. Although this concentration is sufficient to give zero order kinetics with DPN^+, the K_m values for the analogues were not determined, so that the concentration of the analogues used in the tests may not have given the maximum obtainable velocities. Thus, variation in the K_m for various coenzyme analogues in a number of enzyme systems was noted by Kaplan et al. (73).

ATP, DPN^+, DPNH and TPNH were tested for inhibition of TPN-specific isocitric dehydrogenase, and no such effect was found.

TABLE IV

EFFECT OF VARIOUS PYRIDINE NUCLEOTIDES

Pyridine Nucleotide	Relative Activity[a]
	%
A. Oxidized nucleotides	
DPN^+	100
α-DPN^+	0
TPN^+	0
Acetylpyridine-DPN^+	50
Thionicotinamide-DPN^+	7
Deamino-DPN^+	0
Pyridinealdehyde-DPN^+	0
NMN^+	0
B. Reduced Nucleotides	Relative Inhibition[a]
DPNH	100
Acetylpyridine-DPNH	70
Thionicotinamide-DPNH	< 10
Deamino-DPNH	0
TPNH	0[b]

[a] The activity obtained with DPN^+ or DPNH is taken as 100%.

[b] See the text.

All tests done at 25°C. For evaluation of the oxidized nucleotides, each cuvette contained in a volume of 3.0 ml: 100 µmoles of Tris buffer, pH 7.2; 4.0 µmoles of MnCl; 16.0 µmoles of threo-D_sL_s-isocitrate; water, and 1.0 µmole of the nucleotide tested. The reaction was started by adding 25 µgrams of enzyme (specific activity, 500). The reduced nucleotides were tested under similar conditions in the presence of 1.0 µmole of DPN^+.

This enzyme also was not stimulated by FAD, riboflavin-5'-phosphate or riboflavin.

Fluorimetric studies on enzyme-nucleotide complexes. The inhibition by DPNH suggested that the nucleotide might be bound to the enzyme. Binding of reduced pyridine nucleotides to enzymes has been shown in a number of instances to result in an enhancement of the nucleotide fluorescence and a shift of the peak to a lower wavelength (see Udenfriend (74)), an effect first noted for the DPNH-horse liver alcohol dehydrogenase complex (75). Such an effect was obtained also by Langan (68) for TPNH and the TPN-linked isocitric dehydrogenase. In the present studies, similar fluorimetric evidence for binding has been obtained for the DPN-linked enzyme; but this enzyme apparently binds to both DPNH and TPNH (Fig. 10 and 11), in support of the kinetic data given above.

1. Binding to DPNH: Enzyme preparations of different purity were used in the experiments depicted in Fig. 10A and 10B, but the emission peak of DPNH in both instances was shifted from 460 mμ to 444 mμ. The nucleotide fluorescence increased 46% in the presence of 1020 units per ml of the less pure preparation (specific activity, 189) (Fig. 10A) whereas 1420 units per ml of the highly purified enzyme (specific activity, 2490) enhanced the intensity 120% (Fig. 10B) although there was much less protein present. The lack of correlation between enzymic activity and fluorescence enhancement suggests that some quenching may have occurred with the less pure enzyme solution. Alternatively, the

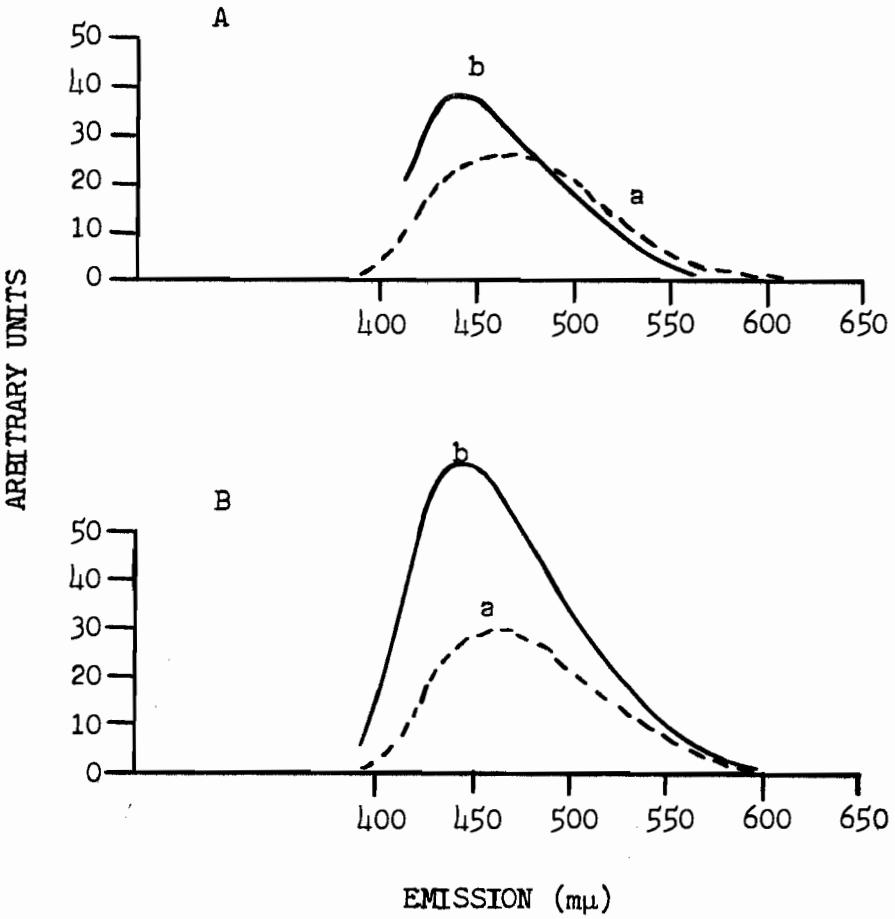

Fig. 10. Comparison of the fluorescence emission spectra of DPNH and the DPNH-enzyme complex.

All solutions contained 0.10 M potassium phosphate, pH 7.2. The excitation wavelength was 360 mμ (uncorrected). The dotted curves (a) are difference spectra obtained by subtracting the fluorescence of the buffer alone from that obtained for 3.3×10^{-6} M DPNH, while the solid lines (b) are the spectra for solutions of protein plus 3.3×10^{-6} M DPNH, corrected for the fluorescence of the protein solution alone. The protein was dialyzed for 2 hours at 2° against buffer, and any precipitate formed was removed by centrifugation. The protein solutions then were allowed to come to 20°. Each cuvette held 3.0 ml of enzyme solution. 0.1 ml of 1.0×10^{-4} M DPNH was then added and the fluorescence spectra immediately recorded. The addition of DPNH resulted in a 3% dilution of the enzyme solution, but the difference spectra are not corrected for this small error. Part A, 5.4 mg of protein per ml (specific activity, 189). Part B, 0.57 mg of protein per ml (specific activity, 2490).

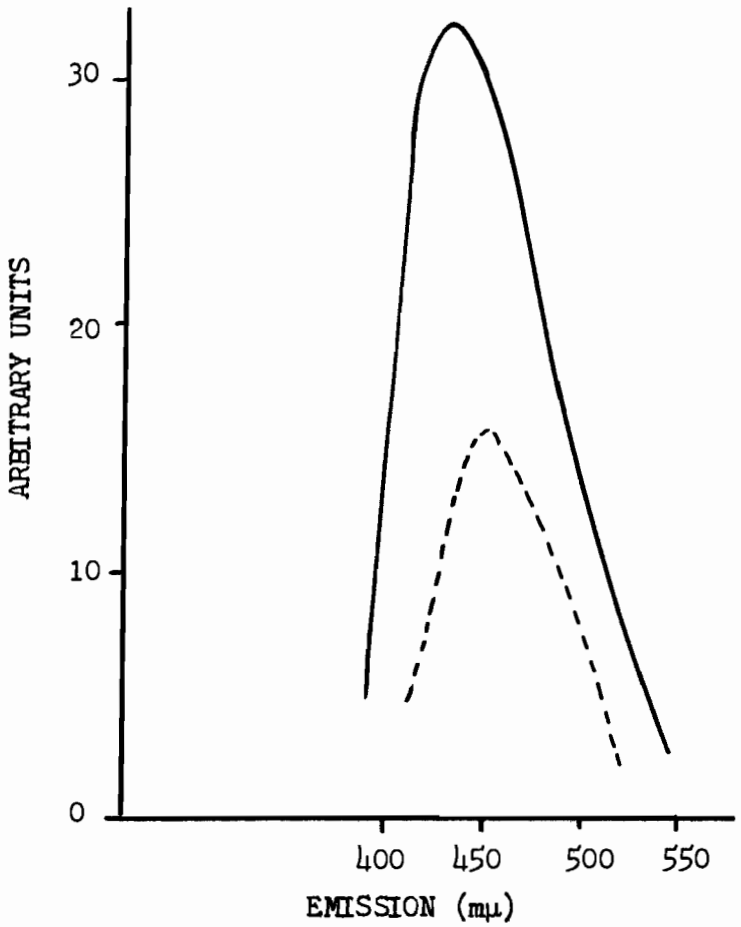

Fig. 11. Fluorescence emission spectrum of TPNH-enzyme complex.

The methods and conditions for obtaining these spectra were the same as described under Fig. 10. A different setting on the voltmeter resulted in greater recorder deflections than obtained in the experiments of Fig. 10. The lower curve represents the difference spectrum obtained by subtracting the fluorescence of the buffer alone from that for a solution of 3.3×10^{-6} M TPNH, while the solid line is the spectrum of the solution of 3.3×10^{-6} M TPNH and enzyme, corrected for the fluorescence of the enzyme solution. Protein, 1.0 mg per ml (specific activity, 600). Some TPN-linked isocitric dehydrogenase activity was present (32 units per ml).

photomultiplier sensitivity may not have been linear over the range of fluorescence intensities encountered. The latter possibility arises because the experiment of Fig. 10A employed large amounts of protein, and considerable fluorescence due to protein had to be subtracted from the spectrum obtained in the presence of both enzyme and DPNH. The correction made for Fig. 10B, however, was quite small.

2. Binding to TPNH: DPN-linked isocitric dehydrogenase seems to form a TPNH-enzyme complex which is discernible in the same way as described above for the DPNH-enzyme complex. As shown in Fig. 11, TPNH fluorescence was augmented about 100% in the presence of enzyme. The peak of fluorescence emission was shifted from 452 mμ to 434 mμ by the presence of protein. These results suggest that although TPNH does not seem to alter the initial reaction rate by itself, it binds to the enzyme and thus forms a complex which is now more sensitive to DPNH inhibition.

The TPNH which was used in the fluorimetric studies was synthesized by enzymic reduction of TPN^{+} with isocitrate in the TPN-specific isocitric dehydrogenase system, and then purified as described in Chapter IV. The DPN-linked enzyme preparation used for the study on TPNH binding still contained detectable TPN-specific isocitric dehydrogenase, but not enough to influence the spectra which were observed.

Since Mn^{++} was not added in any of the fluorescence experiments, DPNH and TPNH seem to bind directly to the protein. Although

there may have been metal ions present in the protein preparations, the amount would have been insignificant since no DPN-linked isocitric dehydrogenase preparations have been noted to be active without added metal ions.

III. DISCUSSION

The finding that DPNH inhibits DPN-linked isocitric dehydrogenase shows that the reduced form of the coenzyme can bind to the enzyme even in the presence of reduced substrate (isocitrate). In this regard, the enzyme differs from TPN-linked isocitric dehydrogenase, which is not inhibited by TPNH (45). Langan's work (68) indicates that the reason for the lack of inhibition by TPNH of the TPN-specific enzyme is that the TPNH cannot bind to the enzyme in the presence of a large amount of reduced substrate. Thus, the ternary complex, enzyme:reduced coenzyme:reduced substrate, is not allowed; and this fact is indicated by the dissociation of the TPNH-enzyme complex in the presence of added isocitrate. In the case of DPN-linked isocitric dehydrogenase, since DPNH reduces the rate of isocitrate oxidation in the presence of an excess of substrate, it is possible that an inactive ternary complex may exist, consisting of enzyme:DPNH:isocitrate. If this were so, the enzyme would differ markedly from other enzymes such as lactic dehydrogenase, which has been studied by Schwert and coworkers (76-79) and has been found not to form "unnatural" ternary complexes in which both substrate and coenzyme are in the reduced form.

Although ternary complexes of the type enzyme:reduced coenzyme: oxidized substrate would be unstable, Schwert's group has been able to demonstrate such complexes by the use of substrate analogues in the case of lactic dehydrogenase (77). Thus, oxalate was used as an analogue of pyruvate, and the enzyme:DPNH:oxalate complex identified fluorimetrically (77). Since these workers found that oxamate was an analogue of lactate, it was not surprising that the enzyme:DPNH:oxalate complex was dissociated by addition of oxamate. Theorell and Langan (80) likewise found that malic dehydrogenase could form a ternary complex with DPNH and D-malate, but not with DPNH and its natural substrate, L-malate. Theorell's group (81,82) has studied the complexes of liver alcohol dehydrogenase and also found that in general, "unnatural" ternary complexes could not be formed either with DPNH and alcohol, or with DPNH and an enzyme inhibitor competitive with alcohol (and analogous to alcohol) such as certain fatty acid amides. Only at extremely high alcohol concentrations was there fluorimetric and kinetic evidence that the complex, enzyme:DPNH:alcohol, had formed (82). Winer and Schwert (83) found that glyceraldehyde 3-phosphate dehydrogenase could not complex with both DPNH and reduced substrate simultaneously, although they obtained some fluorimetric evidence for such a complex with glutamic dehydrogenase. However, Frieden (84) has found that glutamic dehydrogenase may bind DPNH at sites different from the catalytically active site. It seems then, that the lack of inhibition of many dehydrogenases by their reduced coenzymes may

be due to the fact that reduced substrate prevents binding by these coenzyme forms, in spite of the lack of kinetic evidence to indicate strict competition between substrate and coenzyme for the same site on the protein. Thus, DPN-linked isocitric dehydrogenase may be an exceptional case, and DPNH inhibition may not be just a fortuitous finding but rather may be part of a built-in mechanism for the control of enzymic activity under physiological conditions.

The following points may be brought out concerning the inhibition by ATP. Plaut and Plaut (21) found that oxidation of citrate by cardiac mitochondria was increased either by decreasing the amount of ATP in the suspending medium and adding NMN^+ or DPN^+. While other interpretations of these effects undoubtedly are possible, one could attribute such stimulation of citrate oxidation to the removal of ATP inhibition of DPN-linked isocitric dehydrogenase and to reversal by DPN^+ of inhibition by endogenous DPNH. Since the present results show that the enzyme is not directly affected by NMN^+, possibly the effect this nucleotide has of stimulating mitochondrial citrate oxidation is indirect and depends on a secondary reaction, which would also remove ATP and increase DPN^+:

$$ATP + NMN^+ \rightleftharpoons DPN^+ + PP_i$$

Kornberg (85) has found such an enzyme, designated DPN-pyrophosphorylase, in yeast and liver, but Hogeboom and Schneider (86) found that the enzyme was mainly nuclear, at least in liver. Whether such activity exists in cardiac mitochondria is not known. Preiss

and Handler (87) have noted this enzyme in erythrocytes, which contain no nuclei, and found it to be identical to the one catalyzing the analogous reaction involving nicotinic acid mononucleotide and desamido-DPN^+. DPN^+ synthesis, in general, apparently involves not NMN^+ but nicotinic acid mononucleotide (87,88).

The stimulation by ADP was an unexpected finding. Only one other enzyme has been definitely established to be stimulated by ADP: Frieden (89) found that glutamic dehydrogenase of beef liver was markedly stimulated by ADP. (Kornberg and Pricer (4) reported that ADP could replace 5'-AMP for activation of yeast DPN-linked isocitric dehydrogenase, although Kornberg (90) later apparently denied this.) The present results indicate that ADP markedly lowers the K_m for isocitrate and changes the pH optimum towards a more physiological range. Since many related nucleotides had no such activating effect, one suspects that ADP stimulation may play some <u>in vivo</u> role. The enzymic rate at pH 7.2 at low concentrations of isocitrate, say, 1×10^{-4} M, is barely measureable in the absence of ADP, being only 1-2% of the rate in the presence of the nucleotide; and there is evidence that under physiological conditions, isocitrate concentration may be in a range where the ADP effect is very pronounced. Although direct data for intramitochondrial isocitrate concentration are apparently not available, the following considerations suggest that it is low. Bellamy (91) estimated the content of citrate in liver mitochondria

to be about 5 μmoles per gram. The isocitrate concentration would then be less than 3×10^{-4} M if one assumes that the aconitase-catalyzed equilibrium between the tricarboxylic acids obtains (92,93). Thus, under physiological conditions, DPN-specific isocitric dehydrogenase may be virtually dependent on ADP, which could then be regarded as a necessary prosthetic group.

The fact that ADP influences the K_m's of metal ions suggests a mechanism for increasing the efficiency of control of enzymic activity under physiological conditions. Since isocitrate concentration in mitochondria may be so low as to make the enzyme virtually dependent on ADP, this dependency may be augmented if magnesium and manganese ions in mitochondria are also in the range where binding is influenced by ADP. No data are apparently available for the cardiac mitochondrial content of these ions, but Thiers and Vallee (94) have determined the metal content of rat liver mitochondria by emission spectroscopy. Their results show that there are 3×10^{-3} and 3×10^{-5} moles of Mg^{++} and Mn^{++}, respectively, per liter of mitochondria. Since compartmentalization of these ions undoubtedly occurs, and since the amount of aqueous phase available to these ions is not known, it would obviously be speculative to say that these figures for Mg^{++} and Mn^{++} represent the concentrations in the area of the mitochondria where isocitric dehydrogenase is located. However, if Mn^{++} were the metal ion used in vivo by this enzyme, the figures of Thiers and Vallee (94), in conjunction with the values of K_m for Mn^{++} in the presence and

absence of ADP obtained in these studies, would suggest that under physiological conditions, the enzyme has little activity unless activated by ADP so as to be able to interact with Mn^{++} at the catalytic site. On the other hand, if Mg^{++} were the actual cofactor utilized, there may be enough metal ion present in mitochondria to saturate the binding sites without ADP.

Another factor should be considered in the evaluation of metal binding. The K_m values for Mg^{++} and Mn^{++} were obtained in the present study in experiments where isocitrate concentration was saturating. Hathaway and Atkinson (95) have found that the K_m for metal ion in the case of yeast DPN-specific isocitric dehydrogenase <u>increases</u> with decreasing isocitrate concentration. In other words, the K_m value for isocitrate was not independent of the concentration of metal ions, and <u>vice versa</u>. In the case of the beef heart enzyme, the present K_m values for isocitrate were all obtained in the presence of a large excess of Mn^{++}. It is possible that higher K_m values for isocitrate would be found in the presence of lower Mn^{++} concentrations, and that higher K_m values for Mn^{++} or Mg^{++} would be found in the presence of lower isocitrate concentrations. It is conceivable, then, that the enzyme may be even more dependent on ADP than the present data indicate.

Several other enzymes have been found to be activated by nucleotides or phosphate esters. In some cases, it has been said that the enzyme is <u>absolutely</u> dependent on the activator for activity, whereas in other cases the enzyme has significant

catalytic power without the activator. DPN-specific isocitric dehydrogenase can be said to have a specific and almost absolute requirement for ADP at low concentrations of isocitrate. At very high isocitrate concentrations, however, ADP stimulation might not even be detectable. Whether the dependency of other enzymes on their activators has the same relationship to substrate concentration is not certain, but there is at least one case in which an apparently absolute dependency on an activator turned out to be due to measurement of enzymic activity in the presence of low substrate concentrations; namely the case of yeast DPN-linked isocitric dehydrogenase. Kornberg and Pricer (4) measured enzyme activity in the presence of 1.7×10^{-4} M threo-$D_s L_s$-isocitrate (equivalent to 8.3×10^{-5} M of the active isomer) and found essentially absolute requirement for 5'-AMP. This concentration of isocitrate was sufficiently high to obtain maximum rates with the TPN-linked isocitric dehydrogenase system, which was known to have a very high affinity for substrate (K_m, 2.6×10^{-6} M for the pig heart enzyme (17)). However, a recent preliminary communication by Atkinson and Hathaway (69) reports that the DPN-linked isocitric dehydrogenase of Kornberg and Pricer is not dependent on 5'-AMP when the substrate concentration is higher. In fact, the effect of 5'-AMP on this enzyme seems to be exactly analogous to the results found in the present study for ADP and heart DPN-linked isocitric dehydrogenase and reported in 1962 (69); i.e., to increase the apparent affinity for substrate.

The activation of heart DPN-linked isocitric dehydrogenase is, as shown above, catalytic in nature, since the nucleotide does not appear to be consumed in the enzyme-catalyzed reaction, and is comparable to certain other cases of activation. 5'-AMP has long been known to be essential for the activity of phosphorylase b (97,98,99). 2'-AMP has been found to stimulate bacterial pyridine nucleotide transhydrogenase (100). A heat stable, dialyzable factor was found to activate liver dephosphophosphorylase kinase (101), and the factor was later identified as 3',5'-cyclic-AMP (102). Activation of the kinase has in fact been made the basis of a sensitive assay for this nucleotide (103). 3',5'-cyclic-AMP also has been found to stimulate phosphorylase b kinase of muscle (104). This nucleotide was likewise found to activate Fasciola hepatica phosphofructokinase and to decrease the Michaelis constant for fructose 6-phosphate (106). Cormier (106) has identified 3',5'-diphosphoadenosine as the cofactor in the luminescence reaction of the sea pansy, Renilla reniformis; but in this instance, the nucleotide may be reacting with luciferin to give a heat-labile intermediate. 5'-AMP activation of A. niger DPN-linked isocitric dehydrogenase (30) has already been mentioned. Beef liver glutamic dehydrogenase, which is affected by a number of nucleotides is markedly stimulated by ADP and also by 5'-AMP and adenosine to a lesser extent, when DPNH is used as coenzyme (89). ADP also favors aggregation of subunits of the enzyme (89) and protects it from inhibition and disaggregation by steroids (107). On the other

hand GTP is inhibitory (108). It has been postulated that nucleotide stimulation of glutamate dehydrogenase involves configurational changes which also favor aggregation of catalytically active subunits (109,110). Muntz (111) discovered that dog brain 5'-adenylic deaminase activity was stimulated by ATP. The purified deaminase was found to be completely dependent on catalytic amounts of ATP, which did not partake in any exchange reaction with 5'-AMP (112). Ox brain 5'-adenylic deaminase was also stimulated by ATP; but in contrast to the dog brain enzyme, considerable activity was present in the absence of ATP (113). ATP has been shown to be a required cofactor for a 5'-AMP hydrolase of Azotobacter vinelandii (114). The enzyme splits 5'-AMP into adenine and ribose 5-phosphate, and experiments involving isotope-labelled nucleotides showed no interconversion between ATP and the compounds involved in the enzymic reaction. ATP was replaceable by adenosine tetraphosphate, pyrophosphate, and tripyrophosphate, but not by a number of other nucleoside triphosphates. Nishimura et al. (115) have found that aspartic acid β-decarboxylase from C. perfringens contains tightly bound pyridoxal 5'-phosphate and yet requires added pyridoxal 5'-phosphate or an α-keto acid such as pyruvate for activation. K_m for the activating B_6 was found to be 0.9×10^{-5} M, and the co-factor could be dialyzed off readily; whereas enzyme-bound B_6 could not be removed and had to be assayed microbiologically after destroying the protein. DPN^+ apparently is required for certain partial reactions of glyceralde-

hyde-3-phosphate dehydrogenase even though the nucleotide is not consumed (110). TPNH was found to be required for the detritiation of α-ketoglutarate-β-T catalyzed by TPN-linked isocitric dehydrogenase (59). DPN^+ is needed in catalytic amounts by the UDP Gal-4-epimerase of calf liver although there is no evidence to indicate the experience of oxidized intermediates in the reaction (117); the enzyme had a K_m of 2×10^{-7} M for DPN^+ and DPNH was inhibitory. Gerhart and Pardee (118) have recently reported stimulation of the purified aspartate transcarbamylase of Escherichia coli by ATP or dATP; the stimulation by these nucleotides under their standard assay conditions amounted to 80% and 62%, respectively. ATP lowered K_m for substrate, but it was not determined whether or not the enzyme would show apparent dependency on ATP at very low substrate levels.

Aside from those instances already mentioned, other cases are known in which enzyme activation is accompanied by a decrease in K_m for substrate. Thus, Glaser and Brown (119) found that the particulate chitin synthetase of Neurospora crassa was stimulated by N-acetylglucosamine, resulting in a decrease in K_m for UDP-N-acetylglucosamine. K^+ activation of carbamylphosphate synthetase also resulted in lower Michaelis constants for NH_4^+, ATP, and acetylglutamate (121). The activation of UDPG-glycogen transglucosylase by glucose 6-phosphate is due chiefly to a lowering of the K_m for UDPG (121-124). Glaser and Brown (125)

found that Mg^{++} lowers the K_m values for glucose 6-phosphate and TPN^+ in the yeast glucose 6-phosphate dehydrogenase system ("Zwischenferment").

This review of other cases of enzyme stimulation suggests that the reduction of K_m for substrate or coenzyme by an activator may be a more common phenomenon than previously realized. It is clear that many of the cases of enzyme activation have not been studied from the standpoint of whether or not the K_m values are affected.

CHAPTER III

PURIFICATION OF DPN-LINKED ISOCITRIC DEHYDROGENASE OF BOVINE HEART

During the course of the present studies on the DPN-linked isocitric dehydrogenase of bovine heart, several methods of purification were used. Plaut and Sung (5) gave procedures for preparation of beef heart mitochondrial acetone powder, as well as extraction and purification of the enzyme by ammonium sulfate fractionation and calcium phosphate gel treatment. These methods provide amounts of enzyme which are sufficient for most purposes. However, in view of the interaction of the enzyme with nucleotides described in the preceding chapter, it was obvious that large amounts of highly purified protein were desirable in order to facilitate further studies on activation and inhibition.

Plaut and Sung (5,51) achieved a 40-50 fold purification of the initial phosphate buffer extract of beef heart mitochondrial acetone powder in a procedure involving treatment with calcium phosphate gel, ammonium sulfate fractionation, and precipitation chromatography on a starch-Celite column. Their procedure involved only small amounts of enzyme, and less than 1 mg of protein having a specific activity of 1410 units per mg, by the "old assay",

was obtained. The specific activity would correspond to about 2360 units per mg by the assay presently used, in which the reaction is run in the presence of ADP and at pH 7.2 (for enzyme determinations, see Chapter II). The amount of protein obtained by Plaut and Sung was sufficient for experiments which established the stoichiometry and the irreversibility of the enzymic reaction. However, it was noted that the enzyme was highly unstable, so that all purification procedures had to be performed in a single day. Thus, for all practical purposes, enzyme had to be purified daily for kinetic studies.

The present studies on the purification of DPN-linked isocitric dehydrogenase were also hindered at first by the marked lability of the enzyme. Instability has also been noted in the corresponding enzymes of yeast and A. niger (4,30). Instability, plus the fact that there is only a small amount of the DPN-linked enzyme relative to the amount of TPN-specific enzyme in most tissues, undoubtedly has discouraged other workers from investigating DPN-linked isocitric dehydrogenase. However, methods have now been found for stabilization of the beef heart enzyme. Because these methods removed the pressure of time, it was possible to develop improved purification procedures yielding relatively large amounts of enzymic activity.

This chapter will describe various preparative procedures used in exploratory experiments as well as more detailed purifications used to yield enzyme for more exact measurements. Because the

detailed procedures have yielded very pure enzyme, it has been possible to do ultracentrifugal analyses and to assign an approximate molecular weight of 3 or 4 x 10^5 to the enzyme.

I. MATERIALS AND METHODS

Reagents. Acetone was redistilled prior to use. Aluminum hydroxide gel was prepared by the method of Dixon and Webb (126). Calcium phosphate gel was made according to Swingle and Tiselius (127). 2,3-dimercaptopropanol was obtained from Eastern Chemical Corp., Newark, N. J. Phosphocellulose as Cellex P (0.7 meq/gm) from BioRad Laboratories, was distributed by California Corporation for Biochemical Research. All other reagents used were as described in Chapter II.

Preparation of acetone powder. Fresh beef hearts were purchased from the Cudahy Packing Co., North Salt Lake City, Utah, and packed in ice within a half hour of the kill. After trimming the muscle of fat and ligamentous material, the hearts were cut into 1 inch cubes. At this point, the tissues could be stored for at least 4 weeks at -15° for subsequent thawing and use, or could be utilized immediately. The muscle was homogenized in Waring blendors after suspending in a solution containing 0.25 M sucrose and 0.03 M K_2HPO_4 in a ratio of 150 gm of muscle to 400 ml of suspending medium. The homogenate was centrifuged in an International centrifuge, model PR-2, for 10 minutes at 600 x g, in a No. 276 head. The supernatant solution was then freed of

floating fat particles by passage through a double layer of cheese cloth. Dilute acetic acid was added until the pH was reduced to 5.5. The solution was then centrifuged at 1800 x g for 20 minutes and the residue taken up in a small amount of 0.25 M sucrose and centrifuged for 30 minutes at 4000 rpm in a No. 845 head. The residue was then mixed with 5 volumes of acetone at -10° in a Waring blendor and the precipitate collected by centrifugation. The procedure was performed twice more, and the residue was then dried in vacuo at room temperature. 3 kg of heart cubes yielded about 30 gm of mitochondrial acetone powder. The powder was stored in a freezer at -10°.

This procedure for making acetone powder is a slight modification of that of Plaut and Sung (5). Because acidification with acetic acid was extended to pH 5.5 rather than pH 5.8-5.9 (5), greater activity yield was obtained although the crude extract of the resultant acetone powder had lower specific activity.

II. RESULTS

Methods for rapid preparation of partially purified enzyme. These methods differ from those described by Plaut and Sung (5) mainly in the use of 0.1 M potassium phosphate, pH 7.2, to extract the mitochondrial acetone powder, instead of 0.01 M potassium phosphate buffer, pH 6.5. For completeness, these methods are detailed as follows:

A. 1 gm of acetone powder mixed with 20 ml of 0.10 M potassium phosphate buffer, pH 7.2, in a Potter-Elvehjem homogenizer.

The mixture was centrifuged at 18,000 x g for 15 min. in an International PR-2 centrifuge equipped with a high speed attachment and a No. 296 head. The supernatant solution was then fractionated by the slow addition of saturated ammonium sulfate solution, and the material precipitating between 0.4 and 0.5 saturation was collected by centrifugation and dissolved in 5 ml of 0.01 M potassium phosphate buffer, pH 7.2. The specific activity of enzyme thus prepared was about 50-100 units per mg.

Although none of the quantitative data cited in this Thesis were obtained with enzyme which was this crude, these preparations were useful in exploratory experiments involving inhibition and activation by nucleotides.

B. Treatment with calcium phosphate gel resulted in more purified enzyme solutions. 80 ml of extract of mitochondrial acetone powder were stirred with calcium phosphate gel (2.2 mg of gel per mg of protein), which adsorbs the enzyme quantitatively. The gel was collected by centrifugation and washed successively with 80 ml of 0.1 M potassium phosphate, pH 6.5, and 60 ml of 0.6 saturated ammonium sulfate solution. The enzyme was then eluted from the gel with 30 ml of 0.3 saturated ammonium sulfate solution. The concentration of ammonium sulfate was determined by a modification of the Nessler method (60), and the concentration was raised to 0.4 saturation by the addition of saturated ammonium sulfate solution. The precipitate was discarded. The enzyme was precipitated by raising the saturation level to 0.5 by further addition

of saturated ammonium sulfate solution. The protein was collected by centrifugation and dissolved in 5 to 10 ml of 0.01 M potassium phosphate buffer, pH 7.2. Enzyme thus prepared had a specific activity of 500 to 1000 units per mg and was useful for obtaining data cited in some of the figures and tables of Chapter II as well as for the experiments of Chapter IV. Specific activities higher than 1000 were obtained only through more elaborate methods described below.

Stability. When the enzyme was suspended in a medium with an ionic strength of about 0.1 at a concentration of approximately 5 mg/ml, it was noted that over half of the enzymic activity was lost within 24 hours. An example of this decay is depicted in Fig. 12 (bottom curve). It can be seen that under such conditions of a rapidly diminishing activity no effective purification scheme could be devised if it required more than a day or two to perform. After it was found that ADP exerted a stimulatory effect, ADP was tested for stabilization of the enzyme. The results are shown in Fig. 12. ADP did indeed stabilize the enzyme, even in solutions of relatively low ionic strength. Although Fig. 12 shows that 5×10^{-3} M ADP was more effective than 1.6×10^{-3} M ADP, the enzyme used for this storage experiment was shown to contain adenylate kinase, which catalyzes the dismutation, $2ADP \rightleftharpoons ATP+AMP$. The actual amount of ADP which was present is, therefore, uncertain. In other experiments, it was shown that relatively crude preparations of enzyme, with a specific activity of about 300 or less, could also

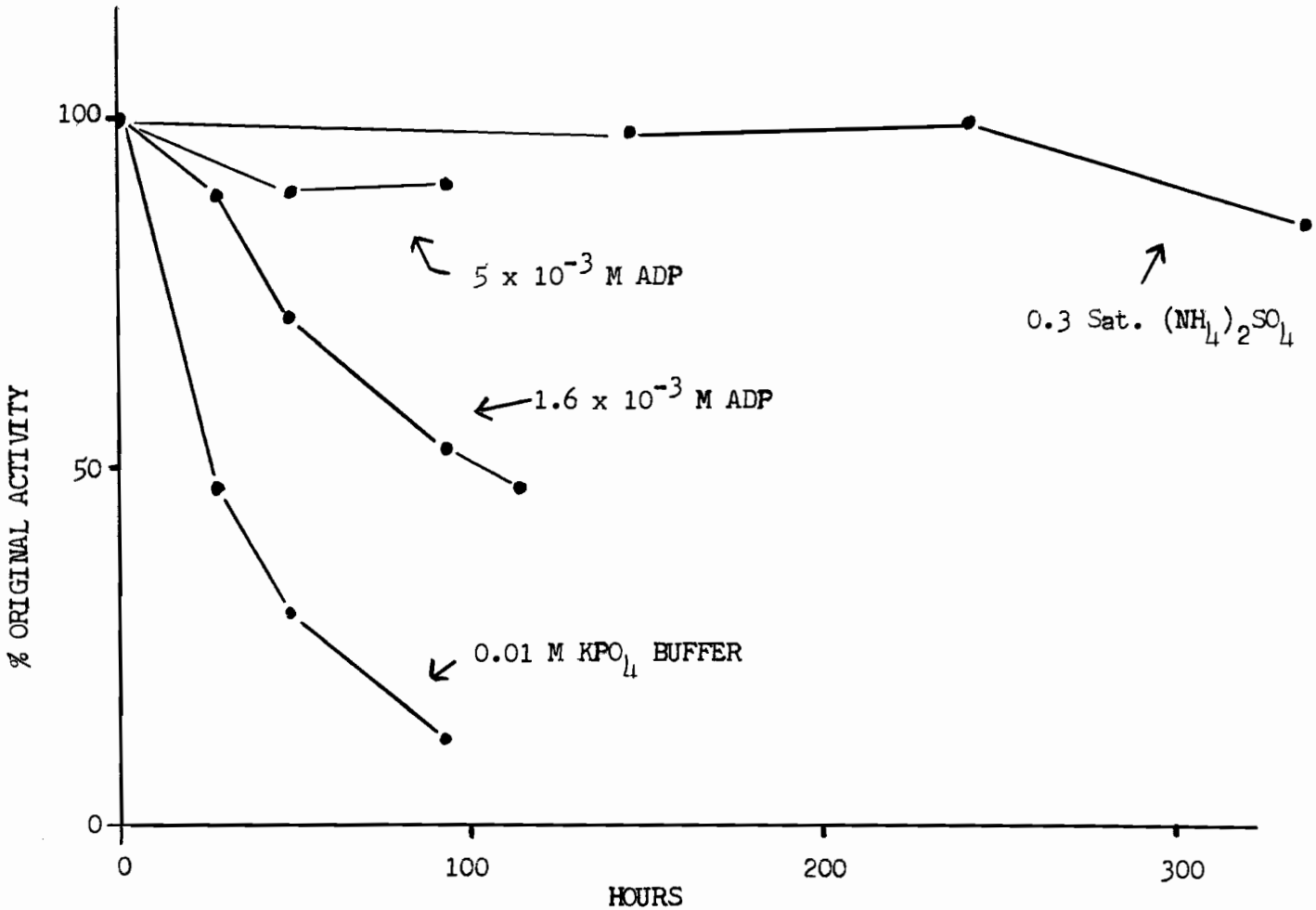

Fig. 12. Activity of DPN-linked isocitric dehydrogenase as a function of time under various conditions of storage.

be stabilized by ATP. However, when ATP was incubated with the enzyme and the solution chromatographed on paper in the system of Krebs and Hems (63), a large spot corresponding to ADP was seen, a finding which indicated that ATPase activity was present. Thus, the actual stabilization might have been due to ADP. A subsequent experiment apparently precluded stabilization by ATP: When highly purified enzyme (specific activity, 3200) which had no measurable ATPase activity was stored with ATP, no stabilization occurred. However, this preparation was stabilized by ADP. Whether the same specificity for ADP which was found in kinetic experiments would be found for enzyme stabilization is uncertain, since other nucleotides were not systematically tested for stabilizing ability.

Certain enzyme preparations purified with calcium phosphate gel absorption and elution (5) seemed remarkably stable. It seemed possible that this procedure removed an inactivator, such as a proteolytic enzyme. However, this possibility was ruled out when small aliquots of crude enzyme were added to the gel-purified preparations, and the enzyme of the mixtures continued to be stable. It was then noted that the stable preparations were made by eluting the enzyme from calcium phosphate gel with 0.3 saturated ammonium sulfate solution. The resulting enzyme solution was actually about 0.28 saturated in ammonium sulfate. Subsequently, it was found that DPN-linked isocitric dehydrogenase was quite stable in 0.2 to 0.3 saturated ammonium sulfate solutions and could be stored

thus for at least 4 weeks with negligible loss of activity (see Fig. 12). However, when the ammonium sulfate concentration of the solutions was brought to 0.4 saturation or higher, enzyme starts to precipitate and seems much less stable as the suspended precipitate. However, if the precipitated enzyme was centrifuged down and stored beneath 0.5 to 0.6 saturated ammonium sulfate solution, activity was again stabilized.

Since the precipitated enzyme was less stable when not centrifuged down, it is possible that precipitation enhances sensitivity to oxidation and inactivation by dissolved air. In this regard, it has been noted that nitrogen purging of buffer solutions results in greater recovery of activity when enzyme has to be dialyzed for long periods of time.

The enzyme was not stabilized in dilute solution (ionic strength about 0.1) by Mg^{++} (1.3×10^{-3} M), citrate or threo-$D_s L_s$-isocitrate ($1\text{-}2 \times 10^{-3}$ M) with or without Mg^{++}; nor by 2,3-dimercaptopropanol (3.3×10^{-3} M). No stabilization was afforded by 0.2% (w/v) gelatin or 0.1% (w/v) bovine serum albumin.

Choice of acetone powder. At one time, whole heart acetone powder seemed more convenient than mitochondrial acetone powder, was made, using pig heart, and the results summarized in Table IV. While the yield of activity from whole heart acetone powder was about 75% greater than from mitochondrial powder, the specific activity was at least ten times lower. The results are quite similar to those reported for guinea pig heart acetone powders (5),

TABLE V

YIELDS OF DPN-LINKED ISOCITRIC DEHYDROGENASE FROM ACETONE POWDERS OF HEART

Source of acetone powder	Yield of powder[a]	Activity[b]	Total[c]	Specific activity[d]
Mitochondria	12	1600	19,200	20
Whole heart	190	180	34,200	1-2

[a]Gms obtained from 1 kg of cubed beef heart.

[b]Expressed as units per gram of acetone powder.

[c]Expressed as units obtained per kg of beef heart.

[d]Expressed as units per mg of protein in the extract, made by dispersing 1 gm of powder in 20 ml of 0.01 M KPO_4, pH 7.2 and discarding insoluble material.

The acetone powder of beef heart mitochondria was made according to Plaut and Sung (5). Whole heart acetone powder was made by homogenizing heart muscle with acetone in the proportion of 150 gm muscle to 450 ml acetone (18). The solid residue was collected by centrifugation at -10° and rehomogenized twice more with the same volume of acetone. The residue was dried under vacuum.

and again confirm that the enzyme is mitochondrial. The difference in yield can be explained mainly on the basis of the inevitable loss of mitochondria during the isolation procedure. (However, it is not known if the enzyme occurs in the nucleus also.) Whole heart acetone powder was not employed for the preparation of the enzyme because the initial extracts had a rather low specific activity and the large amount of powder required extraction with, and handling of, large volumes of buffer.

Beef heart seemed to be a convenient source, although pig heart mitochondrial acetone powder was equally active and probably would have served as well.

Extraction of acetone powder. The enzyme seemed most stable in dilute solutions at pH 7.0-7.2, so extraction of acetone powder was made with buffer adjusted to this pH range. The optimum molarity of the extraction buffer was investigated in terms of the yield and specific activity of the resultant extract. The following results were obtained, when crude extract was prepared as in Table IV:

Buffer used for extraction	Crude Extract	
	Activity (μ/ml)	Specific activity (μ/mg)
0.01 M potassium phosphate, pH 7.0	52	48
0.10 M potassium phosphate, pH 7.0	70	40
1.0 M potassium phosphate, pH 7.0	42	15

0.10 M potassium phosphate buffer was routinely used for extraction of acetone powder because it yielded the largest amount of activity and a fairly good specific activity.

Survey of Purification Methods. Attempts to purify the DPN-linked isocitric dehydrogenase were made employing a variety of methods which had been successful in other cases of protein purification. Some of these methods were capable of purifying the enzyme, but only those methods were ultimately adopted which resulted in high recovery of enzyme activity. Yield was especially important

because of the realtively low amount of enzyme present in tissues. Some of the more promising procedures tried with this enzyme are as follows:

1. Cation exchange resins. A number of experiments in which CM-cellulose was added to dialyzed enzyme solution in 0.01 M potassium phosphate buffer, pH 6.5, showed that the enzyme was not adsorbed strongly to this resin. Since other proteins seemed to be adsorbed under these conditions, enzyme purification by removal of impurities with CM-cellulose appeared feasible. By passing suitably dialyzed protein solutions through 1.0 x 10 cm CM-cellulose columns in 0.01 M potassium phosphate buffer, pH 6.5, it was possible to achieve up to 3.4-fold purification of the enzyme, which eluted in the initial protein peak. The procedure freed the enzyme from the TPN-linked isocitric dehydrogenase which is strongly adsorbed to CM-cellulose (59). Recovery of the DPN-linked enzyme was about 80%. CM-cellulose chromatography was not used routinely for enzyme preparation, because DEAE-cellulose and hydroxylapatite chromatography were more effective.

2. DEAE-cellulose. Batch-type experiments showed that DPN-linked isocitric dehydrogenase adhered to DEAE-cellulose and could be quantitatively eluted with 0.2 M NaCl. Several small column (1.0 x 10 cm) experiments were performed in which resin was equilibrated with 0.005 M potassium phosphate buffer, pH 7.2. Enzyme was dialyzed free of ammonium sulfate by attaching both ends of a dialysis sack to a magnetic stirring bar and spinning

the sack rapidly in 500 volumes of buffer. Determination of ammonia showed that one hour of dialysis, with one buffer change at 30 minutes, was sufficient to lower the ammonium ion concentration to below 0.01 M, a level sufficiently low to allow protein to adhere to DEAE-cellulose. Stepwise washing of the column with 0.025 M NaCl and 0.05 M NaCl, both in 0.005 M potassium phosphate buffer, pH 7.2, resulted in elution of considerable amounts of protein but no enzyme activity. Enzyme was eluted with the NaCl concentration was raised to 0.10 M. Since DEAE-cellulose chromatography gave a high degree of purification and good recovery of activity in these experiments, other columns were run in order to see whether the resin should be used in conjunction with a calcium phosphate gel step, or whether enzyme should merely be fractionated with ammonium sulfate prior to DEAE-cellulose treatment. The results of such experiments are given in Table V. In Experiment 1 of Table V, the crude extract was purified by ammonium sulfate fractionation, most of the enzyme appearing in the protein precipitating between 0.4 and 0.5 saturation ($R_{.4-.5}$). DEAE-cellulose chromatography then produced a preparation with a specific activity of 2670, representing a purification of about 20-fold with respect to the material placed on the column. Experiment 2 shows that DEAE-cellulose achieves only a 2.9-fold purification of protein which has been previously purified with calcium phosphate gel adsorption and elution. The final specific activity resulting from Experiment 2 was 2860, so that DEAE-cellulose purification

TABLE V

PURIFICATION OF DPN-LINKED ISOCITRIC DEHYDROGENASE WITH DEAE-CELLULOSE COLUMNS

Enzyme	Total Units	Protein (mg/ml)	Specific Activity (u/mg)	Purification (Fold)
Expt. 1: Crude extract	6000	2.3	53	1
$R_{.4-.5}$ after dialysis	2400	6.2	130	2.4
DEAE-cellulose column eluate: most active fraction	870	0.19	2670	50.5
Expt. 2: Crude extract	6000	2.3	53	1
Calcium phosphate gel-purified	4000	2.0	1000	18.8
DEAE-cellulose column eluate: most active fraction	3000	1.1	2860	54.9

did not seem to be facilitated by inclusion of a prior calcium phosphate gel treatment step. In subsequent purifications, therefore, calcium phosphate gel was not used.

The small columns of DEAE-cellulose employed in experiments such as those of Table V resulted in 90-100% recoveries of enzyme activity. When such columns were increased in size, however, the longer periods required for dialysis of larger amounts of protein

and for passing larger amounts of solution through the columns resulted in lower recovery of activity. The loss of enzyme activity was probably due to the instability of the enzyme in solutions of low ionic strength.

The use of larger columns of DEAE-cellulose is described below.

3. Acetone fractionation. Since DPN-linked isocitric dehydrogenase was active in acetone powders, it was expected that the enzyme should be stable during acetone fractionation of acetone powder extracts. Fractionation with 90% (v/v) acetone was performed with protein solutions immersed in an ethylene glycol bath at -4° to -6°. 50% to 70% of the enzymic activity was recovered with the protein which precipitated when the acetone concentration was raised from 37% to 52% (v/v). However, although some purification was achieved, the results were inconsistent. In addition, fractionation with acetone was inconvenient since the presence of acetone made simple spectrophotometric determination of protein impossible.

4. Heating. Heating for 15 minutes at 50° causes complete inactivation of DPN-linked isocitric dehydrogenase if it is suspended in phosphate buffer, pH 7.2, at an ionic strength of 0.1. Under these conditions, enzyme activity is still completely lost in the presence of 6.7×10^{-4} M ADP, a concentration of the nucleotide which is in the optimal range for stimulation of enzymic rate.

However, when enzyme is dissolved in concentrated ammonium

sulfate solutions (0.3 to 0.4 saturated), heating at 50° may be carried out for periods up to 60 minutes without loss of activity. The optimal conditions for purification by the use of heating were investigated with enzyme which had been prepared from 20 ml of crude extract of mitochondrial acetone powder. The protein which precipitated on addition of saturated ammonium sulfate between 0.4 and 0.5 saturation was taken up in 10 ml of 0.3 saturated ammonium sulfate solution. Aliquots were heated at 50° for periods of time up to 45 minutes. Rather copious precipitation occurred within 5 minutes. The solutions were rapidly cooled in an ice bath. The precipitates were removed by centrifugation and the enzyme assayed in the supernatants. It was found that a 2-fold increase in specific activity occurred after heating for 15 minutes; but longer periods of heating resulted in no further purification. When the ammonium sulfate concentration was then raised to 0.35 saturation, up to half of the remaining protein precipitated, leaving the enzymic activity in the supernatant. Heating appeared to be a particularly advantageous step in the purification of enzyme, because it was noted that a reproducible <u>increase</u> in enzymic activity occurred, amounting to 25-30%. Enzyme preparations which had thus been heat-activated could not be further activated by heating again at some subsequent time. Other experiments showed that activity was partially destroyed by heating for 15 minutes at 55-60°. Therefore, heating for 15 to 20 minutes at 50° seemed to allow maximum purification with a reasonable safety margin as

regards temperature.

5. Hydroxylapatite chromatography. Hydroxylapatite gel was prepared essentially according to Tiselius et al. (128). Since the enzyme could be purified by adsorption on the calcium phosphate gel, it was no surprise that batch experiments with hydroxylapatite revealed that the enzyme could be adsorbed quantitatively from 0.01 M potassium phosphate buffer at pH 7.0 and eluted with 0.20 M potassium phosphate buffer at the same pH. Columns of hydroxylapatite were then packed by gravity and found to purify the enzyme when protein was eluted by stepwise addition of increasingly concentrated phosphate buffer at pH 7.2. However, considerable difficulty was experienced in obtaining adequate flow rates with certain preparations of hydroxylapatite, so that it was necessary to add a small amount of Celite to the gel. It was also noted that different preparations of hydroxylapatite varied with respect to the strength of adsorption of the enzyme to the gel, since certain batches of adsorbent allowed enzyme elution with 0.2 M NaCl whereas others retained the enzyme even with 1.0 M NaCl.

When hydroxylapatite gel was prepared without the use of magnetic stirring bars, however, it was noted that the characteristics of the gel were more uniform and that the flow rate was much increased. These observations confirm those of Levin (129), who noted that gentle stirring must be employed since the calcium phosphate crystals are rather fragile. With gel prepared only with overhead mechanical stirring, enzyme could be eluted with 0.2 M potassium phosphate

buffer at pH 7.2 but not by 1.0 M NaCl or by 0.10 saturated ammonium sulfate solutions. The flow rate resulting from packing the gel by gravity generally was greater than desired, so that regulation of flow with a constant delivery pump (Mini-pump, Milton Roy Co., Philadelphia) was employed. Details of hydroxylapatite chromatography are given below.

General Procedure for Enzyme Purification.

Unless otherwise stated all procedures were carried out at 2-5°.

1. Extraction - Acetone powder (232 gm) was homogenized in a Waring blendor with 4000 ml of 0.10 M potassium phosphate buffer, pH 7.2. The homogenate was centrifuged at 1000 x g for 20 minutes, and the residue resuspended in an additional 1600 ml of buffer. After centrifugation, the supernatant solutions were combined.

2. Ammonium sulfate fractionation and heat step - Solid ammonium sulfate was added to the extract while the reaction was maintained at pH 7.0-7.4 by addition of solid Na_2CO_3 (approximately 0.1% of the weight of the ammonium sulfate added). The material precipitating between 30% and 50% saturation was mixed with 500 ml of 30% saturated ammonium sulfate solution with a Potter-Elvehjem homogenizer. The suspension was rapidly heated in a water bath and the temperature maintained at 50° for 15 minutes. The suspension was then rapidly cooled in an ice bath and centrifuged. The sediment was discarded.

3. Ammonium sulfate fractionation - Saturated ammonium sulfate solution was added to the supernatant fluid from step 2. The material precipitating between 35% and 45% saturation was collected by centrifugation and stored after suspending in 60 ml of 0.3 saturated ammonium sulfate solution.

4. Dialysis - Saturated ammonium sulfate (8.0 ml) was added to a 20 ml aliquot of the enzyme suspension from step 3. The precipitate was collected by centrifugation and dissolved in 27 ml of 0.005 M potassium phosphate buffer, pH 7.2, containing 0.01 M ATP and dialyzed for two hours with high speed stirring against 2000 ml of 0.005 M potassium phosphate buffer, pH 7.2, which had been purged with N_2 previously. The cloudy precipitate appearing in the protein suspension was removed by centrifugation. The ammonium ion content of the enzyme solution was usually found to be 0.03 to 0.04 M at this point. By slow addition of buffer, the enzyme solution was diluted until the ammonium ion content was 0.02 M.

5. Chromatography on DEAE-cellulose - Portions of the enzyme solution from step 4 were placed on four DEAE-cellulose columns previously equilibrated against 0.005 M potassium phosphate buffer, pH 7.2. About 100 mg of protein was placed on each 2.2 x 12 cm column. A linear gradient was produced by means of a mixing chamber containing 150 ml of 0.005 M potassium phosphate buffer, pH 7.2, and a reservoir containing 150 ml of buffer plus 0.2 M NaCl. A uniform flow rate of 2.2 ml per minute was maintained with a

Mini-pump. 15.0 ml fractions were collected and the tubes with the highest specific activity were pooled. The results of a typical DEAE-cell. column are shown in Fig. 13. An equal volume of saturated ammonium sulfate solution was added to the pooled fractions to precipitate the protein. Following centrifugation, the enzyme was stored as the precipitate beneath the supernatant solution. Prior to the next step, the protein was dialyzed against buffer as in step 4, but without ATP in the dialysis buffer.

6. Hydroxylapatite chromatography - The enzyme was applied to a 2.2 x 12 cm hydroxylapatite column previously equilibrated against 0.005 M potassium phosphate buffer, pH 7.2. It was eluted from the column with a linear gradient system of potassium phosphate buffer, pH 7.2, produced with 150 ml of 0.005 M buffer in the mixing chamber and 150 ml of 0.20 M buffer in the reservoir initially. Although the column had a flow rate of 2.2 ml per minute with a pressure of 20 cm of water, the flow rate was maintained at 1.5 ml per minute with a Mini-pump and 5.0 ml fractions were collected. The fractions with the highest specific activities (3410 to 5520 units per mg of protein) were pooled and the protein was precipitated by adding saturated ammonium sulfate to give 0.6 saturation. The protein was centrifuged down and stored in the centrifuge tube beneath the supernatant fluid. The enzyme was dissolved and dialyzed for ultracentrifugal analysis as described below. The purified enzyme is colorless and has an A_{280}/A_{260} ratio of 1.7.

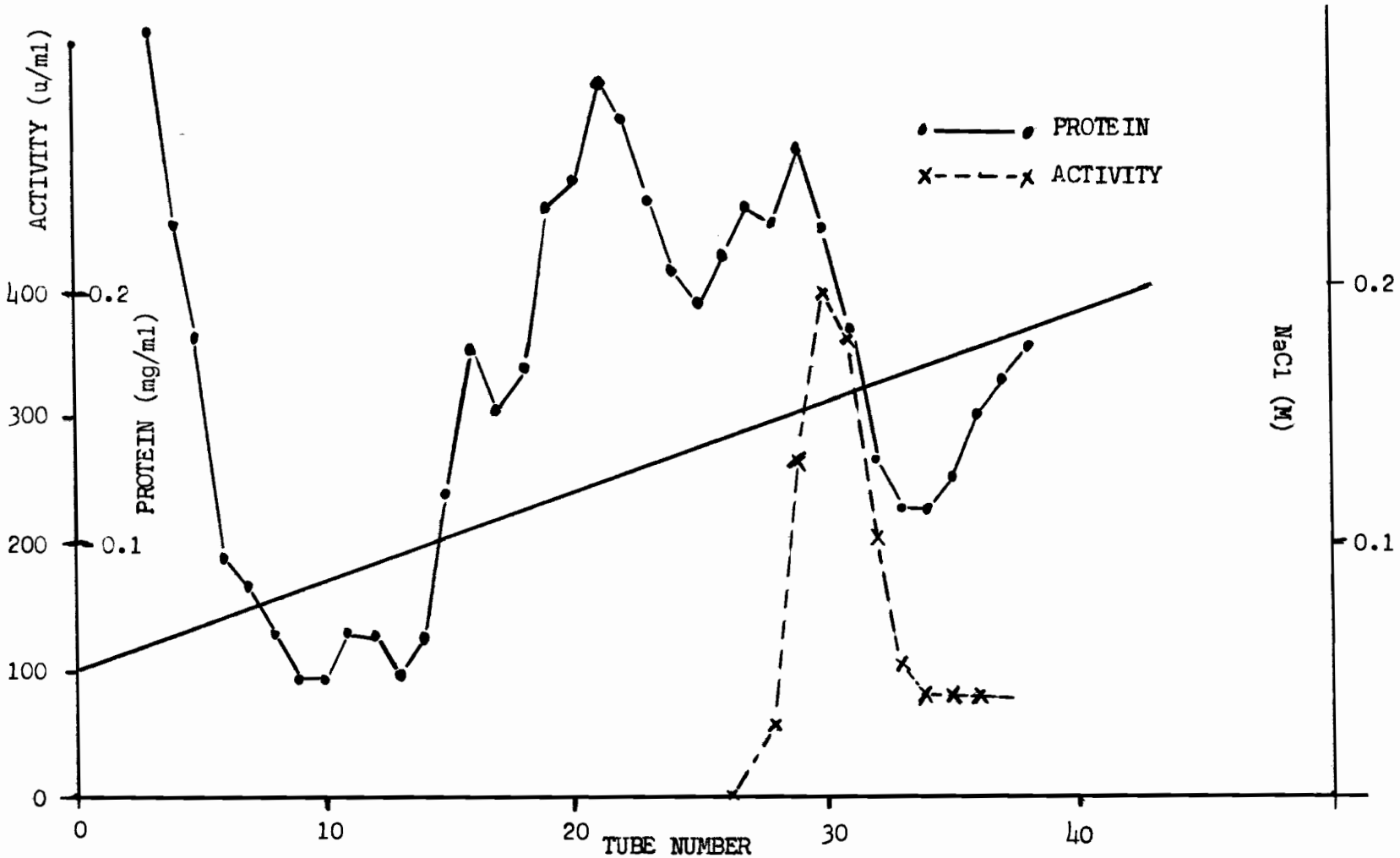

Fig. 13. DEAE-chromatography of DPN-linked isocitric dehydrogenase.

A summary of the purification procedure is given in Table VI.

In this procedure, as noted in Table VI, heating in 0.30 saturated ammonium sulfate results in 29% more activity than present in the initial extract of mitochondrial acetone powder. The DEAE-cellulose chromatography step represents a number of compromises. As noted above, small columns indicated the feasibility of this resin for purification purposes. However, when larger columns were run, it became necessary to use rather fast flow rates in order to avoid exposing the enzyme to low ionic strength solutions for long periods of time. Thus, four separate 2.2 x 12 cm columns were used rather than a single larger column; and in addition, a rapid flow rate of 2.2 ml per minute had to be employed although this is probably too fast for optimal ion exchange separation. These are factors explaining the 47% loss in activity in the DEAE-chromatography step, which also resulted in enzyme with a specific activity of only 1070 units per mg although it had been shown that smaller DEAE-cellulose columns could produce enzyme of greater purity. Hydroxylapatite chromatography, on the other hand, always resulted in higher activity recovery than DEAE-cellulose chromatography; and it seemed that the enzyme was stable when adsorbed onto hydroxylapatite gel. Because of the disadvantages of DEAE-cellulose chromatography, an alternate procedure was developed in which this resin is replaced by a hydroxylapatite chromatography step employing elution of protein with concentrated ammonium sulfate solutions.

TABLE VI

PURIFICATION OF DPN-LINKED ISOCITRIC DEHYDROGENASE FROM BOVINE HEART

Step	Volume ml	Activity units/ml	Total Activity units	Activity Yield %	Total Protein mg	Specific Activity units/mg	Purification fold
1. Crude extract	5,170	44	217,000	100	37,000	6.2	1
2. Ammonium sulfate + heat treatment	525	528	277,200	128	3,680	75	12.5
3. Ammonium sulfate fractionation	62	4,500	279,000	129	2,090	134	22
4. Dialysis*	37	2,280	84,800	100	405	200	32
5. DEAE-cellulose chromatography*	14	3,200	45,080	53	42	1,070	173
6. Hydroxylapatite chromatography*	1.7	19,500	33,200	39	7.4	4,500	727

*One-third of the protein from Step 3 was purified in Steps 4 to 6. The activity yield has been adjusted for the change in amounts.

Alternate Purification Procedure. All the steps were the same as the "General Procedure" above except that steps 4 and 5 were replaced as follows. Saturated ammonium sulfate (8.0 ml) was added to a 20 ml aliquot of the enzyme suspension from step 3, above. The precipitate was collected by centrifugation and dissolved in 50 ml of 0.005 M potassium phosphate buffer, pH 7.2. The solution was placed directly on a 2.2 x 12 cm hydroxylapatite column without dialysis. The column was washed in succession with 50 ml of 0.005 M potassium phosphate buffer, pH 7.2; 100 ml of 0.10 saturated ammonium sulfate solution; and 100 ml of 0.20 saturated ammonium sulfate solution. Washing with 0.10 saturated ammonium sulfate solution caused elution of the colored proteins from the column, so that when enzyme was eluted with 0.20 saturated solution, the eluate was colorless. A diagram of this procedure is shown in Fig. 14. The fractions with the highest specific activity (800 to 1100 units per mg) were pooled and purified as in step 6, above, by means of a second hydroxylapatite column procedure in which protein elution was accomplished by increasing phosphate buffer concentration.

The substitution of hydroxylapatite chromatography for DEAE-cellulose purification has the advantage of eliminating a dialysis step and avoids the need to expose the enzyme to low ionic strength solutions.

In the alternate procedure, the enzyme, after purification on the second hydroxylapatite column and dialysis, has a specific

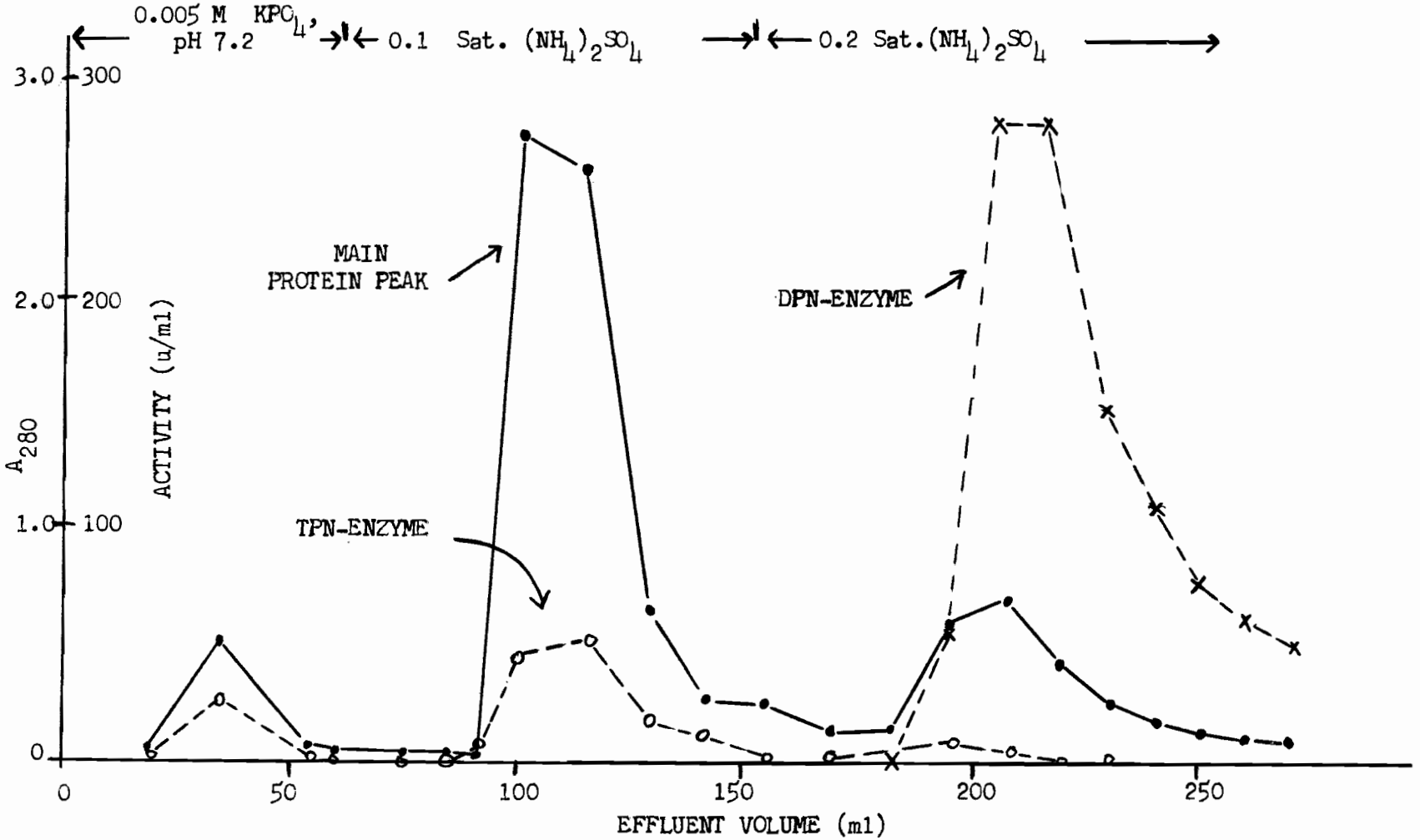

Fig. 14. Hydroxylapatite chromatography of DPN-linked isocitric dehydrogenase.

The dashed lines represent enzymic activity and the solid line indicates protein as determined by absorption at 280 mμ. The data would be more correctly represented as a histogram; the points shown indicate protein and activity in the fractions collected after passage of the effluent volume indicated on the abscissa.

activity of about 4,000 as opposed to 4,500 obtained by including DEAE-cellulose chromatography. The recovery of activity is 80 to 90% compared to slightly over 50% for the DEAE-cellulose step.

Preliminary experiments indicate that further increase in yield may be obtained by a slight modification of the second hydroxylapatite column procedure, as follows. After protein has been adsorbed onto the column, a gradient is produced for elution by means of 150 ml of 0.10 saturated ammonium sulfate in the mixing chamber and 150 ml of 0.10 saturated ammonium sulfate solution containing 0.20 M potassium phosphate buffer, pH 7.2. The enzyme is, therefore, kept in solutions of high ionic strength throughout the entire purification procedure with the exception of the initial extraction of mitochondrial acetone powder.

Ultracentrifugation of purified enzyme[4]. The protein of step 6 (Table VI) of the purification procedure was taken up in 1.0 ml of 1×10^{-4} M ADP and dialyzed for 60 minutes against 1000 ml of 0.10 M potassium phosphate buffer, pH 7.2, containing 1×10^{-5} M ADP. When centrifuged at 59,780 rpm in a Spinco model E ultracentrifuge at 20°, the sample showed one major (about 85%) and

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[4]The author is indebted to Mr. D. M. Brown for performing the ultracentrifugal studies and consultation on the approximate molecular weight of the protein.

one minor (about 15%) component sedimenting at rates of 10.3 S and 4.6 S, respectively. The schlieren pattern is shown in Fig. 15A.

There were several cogent reasons for believing that the DPN-linked isocitric dehydrogenase was associated with the major component (10.3 S): 1) After ultracentrifugation, the top third of the cell contents containing the component sedimenting at 4.6 S was drawn off with a syringe. Although much mixing occurred during this procedure, the fluid at the bottom of the cell had 83% of the activity and a specific activity 3 times higher than the fluid removed. 2) In a separate experiment with Sephadex G-200 gel, a "molecular sieve" capable of holding proteins with molecular weights as high as 200,000, passage of the enzyme was essentially unretarded. In this experiment, a crude extract of mitochondrial acetone powder was placed on a 2.2 x 20 cm column, and it was obvious that most of the colored proteins, probably containing hemoglobin, were markedly retarded by the column, whereas DPN-linked isocitric dehydrogenase activity came out with the initial protein peak. Substances which, like the minor component of Fig. 15A, have a sedimentation constant of 4.6 S would be about the same size as hemoglobin and would have been eluted from the column rather late. 3) Ultracentrifugation was also performed on several other samples of purified enzyme. A sample prepared by the alternate procedure described above, in which DEAE-cellulose chromatography is replaced by another hydroxylapatite column, had a specific activity of about

3200 and appeared on ultracentrifugation to contain a major component (10.3 S) and a minor component (6.2 S), as shown in Fig. 15B. The only component common to the samples of Figures 15A and 15B, is that sedimenting at 10.3 S. Under these conditions of ultracentrifugation, three other samples (total of five samples) with high enzyme activity exhibited the 10.3 S component. 4) If the major component of 10.3 S represents the enzyme, a turnover number can be calculated on the basis of a specific activity of 5520 units per mg and a molecular weight of 300,000 (see below). The calculated turnover number of 8000 moles of DPNH formed per minute per mole of enzyme is a reasonable one, and is over twice the turnover number for the TPN-linked isocitric dehydrogenase of heart: 3,500 moles of TPNH formed per minute per mole of enzyme (18). If either the small 4.6 S or 6.2 S components of Figs. 15 A and 15 B were the enzyme, the turnover number would be extremely high and would be much greater than the catalytic rate of most dehydrogenases.

Molecular weight. The sedimentation coefficient of 10.3 S suggests that the enzyme is a large molecule. Since not enough protein was present to do diffusion studies, the molecular weight can only be guessed at. By comparison with the sedimentation constants of better characterized proteins, the 10.3 S constant of DPN-linked isocitric dehydrogenase leads to a very rough estimate of 300,000 to 400,000 for the molecular weight.

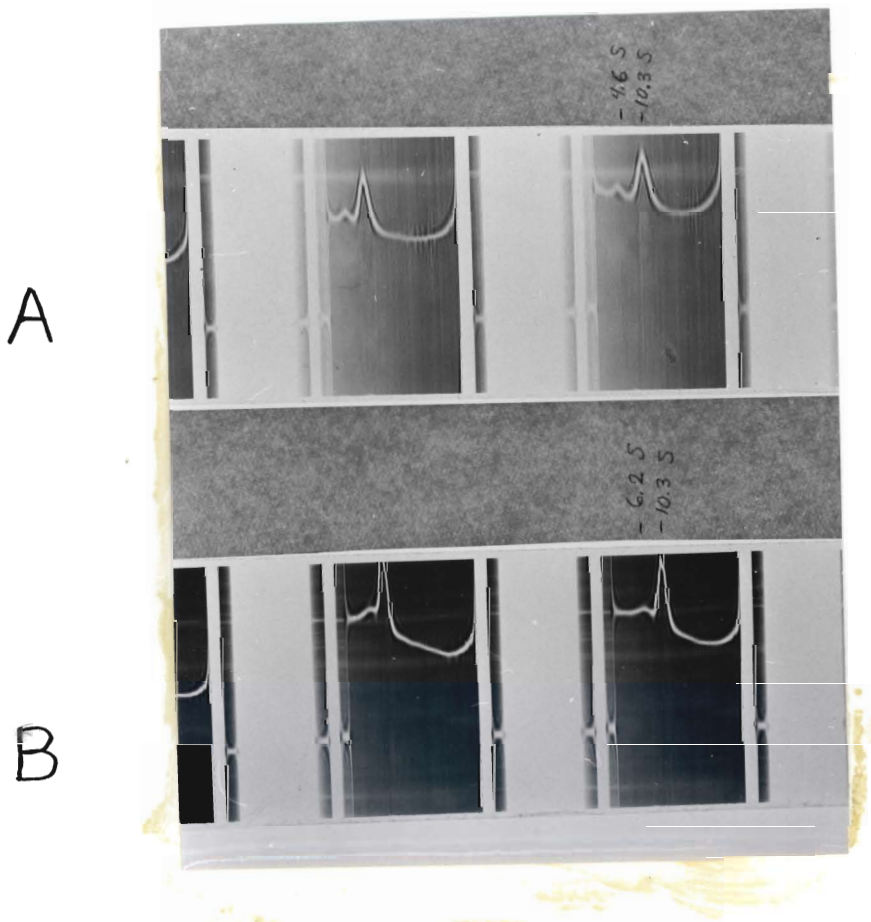

Fig. 15. Schlieren patterns of ultracentrifugal analyses of enzyme preparations.

Part A: The enzyme was prepared according to the "General Procedure" as described in the text. Specific activity, 4500 units/mg. Part B: The enzyme was prepared according to the "Alternate Procedure" as described in the text. Specific activity, 3200 units/mg.

In both A and B, the direction of sedimentation is from left to right. The pictures shown were taken at 24 and 32 minutes following attainment of a speed of 59,780 rpm.

III. DISCUSSION

The pivotal finding reported in this chapter was the stabilization of the DPN-linked isocitric dehydrogenase by concentrated ammonium sulfate solutions. Without a practical method for storing this enzyme, purification, and other studies could not have been performed. It will be of interest to see whether other DPN-linked isocitric dehydrogenases from other sources such as yeast or A. niger (4,30) are also stabilized by solutions of high ionic strength.

Plaut and Sung (5) reported previously that their purification procedure resulted in a preparation with a specific activity of 1410 units per mg. The present preparation (the "general procedure") resulted in a specific activity of 5520 in the most active fraction obtained on hydroxylapatite chromatography, although the pooled fractions had a specific activity of only 4500. The purification is only about 2-fold greater than that obtained by Plaut and Sung (5), since, in the present work, an improved assay method was used which yielded 67% more apparent activity than the old assay method. This estimate of the molecular weight for the DPN-linked enzyme is based merely on comparison of the sedimentation constant (S^{20}_{w}) of 10.3 S with the sedimentation constants of proteins whose molecular weights have been determined more accurately[4]. The small amount of protein available in the present studies precluded studies to determine the diffusion constant or to examine the electrophoretic behavior of the enzyme. No

information is thus available at present on factors such as the shape, degree of hydration of the enzyme molecule, or its partial specific volume.

There is no evidence that there is any flavin attached to the protein. The purified enzyme appears to be colorless, and separate experiments have shown no inhibition of the enzyme by atabrine. Furthermore, no stimulation by riboflavin, FAD, or riboflavin 5'-phosphate occurs.

It is not possible to explain the heat activation of the enzyme. The per cent increase in activity on heating in 30% ammonium sulfate solution is quite reproducibly in the range of 25-30%. One could speculate that heat treatment allows dissociation of some inhibitory substance which cannot recombine with the enzyme in solutions high in ammonium sulfate concentration. Alternatively, ammonium sulfate itself may be the activating agent, which cannot reach the activating site on the protein except after the molecule has been thermally agitated. Activation by ammonium sulfate has recently been noted for pig heart malic dehydrogenase (130).

With regard to stability, the DPN-linked isocitric dehydrogenase of heart is somewhat similar to the enzyme carbamylphosphate synthetase. Metzenberg *et al.* (131) found that the carbamyl phosphate synthetases of dog and beef liver had a half life of only 1 hour at 0° in dilute buffers, at pH 7.4, but were stable in concentrated ammonium sulfate and when adsorbed on hydroxylapatite columns. The stabilization by ammonium sulfate was further investi-

gated by Ravel et al. (132) who showed that it was the sulfate ion which afforded stabilization, since sodium and potassium sulfate were equally effective whereas ammonium carbonate or phosphate was not. These workers also found that the enzyme, which had been inactivated by dialysis against dilute buffer or by exposure to heat, could be reactivated to some extent by sulfate.

In the case of DPN-linked isocitric dehydrogenase, heat inactivation has not been reversed by ammonium sulfate, although prevention of heat inactivation has been recorded. Prevention of heat inactivation by ADP has not been shown in a limited number of experiments, however. Although substrates and cofactors may either enhance or decrease enzyme stability (133), DPN-linked isocitric dehydrogenase stability does not seem to be influenced by DPN^{+}, DPNH, isocitrate, Mn^{++}, or Mg^{++}.

The estimated molecular weight of 300,000 to 400,000 places DPN-specific isocitric dehydrogenase in the same size category as other large mitochondrial enzymes. In the citric acid cycle, succinic dehydrogenase has a molecular weight of about 200,000 (134); α-ketoglutaric dehydrogenase isolated from porcine heart is a complex with a molecular weight of some 2×10^6 (135); fumarase has a molecular weight of 220,000 (136). However, malic dehydrogenase appears to be quite small, although there are apparently several forms of the enzyme (137). Molecular weights have ranged from 20,000 to 65,000 for malic dehydrogenase. Condensing enzyme likewise is small, having a molecular weight of 56,000 (138).

Both beef liver glutamic dehydrogenase (139) and pigeon breast muscle pyruvic oxidase (140) yield products which can enter the citric acid cycle and are large protein complexes with molecular weights of 1×10^6 and 4×10^6, respectively.

It is conceivable that DPN-linked isocitric dehydrogenase may be smaller than 300,000, since there are certain proteins whose molecular weights are smaller than this and have higher sedimentation coefficients than 10.3 S. Thus, glutamic dehydrogenase, said to have a molecular weight of 1,000,000 (139), breaks up under certain conditions into subunits having a presumed molecular weight of 250,000. The sedimentation coefficient of the subunits appears to be about 12 S (89). On the other hand, light scattering data suggest that glutamic dehydrogenase actually has a molecular weight as high as 1.3×10^6 (141). Light scattering data also yields a higher molecular weight for succinic dehydrogenase than do sedimentation-diffusion studies (142). It seems possible that these enzymes, which presumably could have been tightly associated with the lipid-rich mitochondrial membrane, may still be associated with lipid, thereby having higher partial specific volumes than those assumed for purposes of calculations of molecular weight from sedimentation data. Ultracentrifugal data would yield molecular weights which are too low. Since the same possibility exists for the DPN-linked isocitric dehydrogenase, it seems wisest at the present time to estimate the molecular weight to be in the range of 3 to 4×10^5.

CHAPTER IV

STUDIES ON THE HYDROGEN TRANSFER MEDIATED BY DPN-LINKED ISOCITRIC DEHYDROGENASE

Good evidence exists that the DPN- and TPN-linked isocitric dehydrogenases of heart must differ in some fundamental aspects of the catalytic mechanism, because the latter enzyme can use oxalosuccinate as substrate whereas the former cannot. Furthermore, the TPN-specific enzyme catalyzes the synthesis of isocitrate from α-ketoglutarate and CO_2, whereas the DPN-linked isocitric dehydrogenase reaction apparently cannot be reversed (5).

Studies on the mechanism of action of the TPN-linked enzyme have been performed by other workers, who have obtained interesting results in experiments employing hydrogen and carbon isotopes as labels. Thus, Englard and Colowick (143) oxidized isocitrate with the porcine heart TPN-linked isocitric dehydrogenase system in D_2O, isolated the reduced TPN produced, and found that the nucleotide contained no deuterium. This experiment indicated that the hydrogen transfer was a direct one from substrate to coenzyme, without possibility of exchange of hydrogen atoms with the medium. These workers also incubated citrate with aconitase in D_2O to produce labeled isocitrate, which was then oxidized by the TPN-specific isocitric dehydrogenase to yield unlabeled TPNH. This

experiment showed that it must be the α- and not the β-hydrogen of isocitrate which is attacked by the TPN-linked isocitric dehydrogenase. Englard (144) has confirmed these results in experiments in which labeled isocitrate was produced with aconitase, and H_2O, rather than unlabeled citrate, aconitase, plus D_2O. Recently, the TPN-linked isocitric dehydrogenase has been found to be specific for the α-side of the nicotinamide ring (145). This was the first TPN-linked enzyme for which such studies of pyridine nucleotide stereo-specificity were done.

Siebert et al. (19), using C^{14} as label, found that oxalosuccinate, although it could be used as substrate by TPN-linked isocitric dehydrogenase, was not likely to be an obligatory free intermediate in the overall reaction. Thus, only minute amounts of radioactivity were incorporated into an oxalosuccinate pool, either from C^{14}-labeled isocitrate and TPN^+, or from $C^{14}O_2$ and α-ketoglutarate plus TPNH.

Since no analogous data had been obtained for the DPN-specific isocitric dehydrogenase, studies on the hydrogen transfer mediated by the enzyme were undertaken. It was of particular interest to determine whether it was the α- or β-hydrogen of isocitrate which was transferred in the DPN-specific reaction, and whether this transfer was direct and stereospecific. If the enzyme contained flavin, as do other large enzymes related to the Krebs cycle; i.e., succinic dehydrogenase (134) and α-ketoglutaric dehydrogenase (140), it was possible that the hydrogen transfer would not be direct. Studies

were also performed to test the possibility that the DPN-linked enzyme labilized a hydrogen of isocitrate to cause an exchange with the hydrogen of the medium.

The results of the present studies which employed tritium as label, indicate that the transfer of hydrogen from substrate to DPN^+ is a direct one, involves the same hydrogen of threo-D_s-isocitrate as that involved in the TPN-linked reaction, and that the hydrogen is transferred to the α-side of DPN^+. Evidence has also been obtained that the enol form of oxalosuccinate is not an intermediate in either isocitric dehydrogenase reaction, since in both reactions, the β-hydrogen of isocitrate appears to be retained in the α-ketoglutarate produced.

I. MATERIALS AND METHODS

DPN-linked isocitric dehydrogenase with a specific activity of 800-1000 units/mg was prepared by calcium phosphate gel adsorption and elution as described in Chapter III, and stabilized with 1×10^{-4} M ADP. The enzyme solution employed was also 30% saturated with respect to ammonium sulfate.

TPN-linked isocitric dehydrogenase was prepared as described in Chapter II.

Tritiated water, 100 mc/ml, was obtained from New England Nuclear Corp. and distilled before use.

Norit A (acid washed) from Pfanstiehl Laboratories, Inc., was washed with EDTA at pH 6.2 for 48 hours before use. (In

the separation of DPN^+ from glutamate, the latter was adsorbed to Norit A unless the charcoal had been washed with EDTA.)

Crystalline liver L-glutamic dehydrogenase (ammonium sulfate suspension), crystalline rabbit muscle lactic dehydrogenase (Type I), and yeast glucose 6-phosphate dehydrogenase (Type V) were purchased from the Sigma Chemical Co. A unit of glucose 6-phosphate dehydrogenase reduces 1.0 μmole of TPN^+ per minute under standard assay conditions (54).

Crystalline lithium lactate, synthesized by the method of Hillig (146), was a gift of Dr. G. W. E. Plaut. Sodium pyruvate was obtained from Nutritional Biochemicals, Inc. The barium salt of oxalosuccinic acid, synthesized according to Ochoa (147), was a gift of Dr. G. W. E. Plaut. The reagent had been stored at -10° for five years. Oxalosuccinate was obtained as the barium-free solution by treatment with K_2SO_4 and was assayed manometrically in a Warburg respirometer by the aniline citrate decarboxylation method of Edson (148). The barium oxalosuccinate was 40% pure as judged by weight and the manometric data, whereas it had originally been assayed as 70% pure. Presumably, some of the acid had spontaneously decarboxylated to α-ketoglutaric acid.

Silica gel (Merck) columns were prepared according to Brummond and Burris (149).

All other reagents used were the same as those described in previous chapters.

The method of preparing TPNT[5] was a modification of that used by Popjak et al. (151). To 0.30 ml of T_2O containing 52 mc/ml were added 3.9 mg of $NaHCO_3$, 5.05 mg of TPN^+ (6.0 μmoles), and 3.60 mg of $Na_2S_2O_4$. The mixture stood at 25° for 45 minutes and then was lyophilized in a closed system, so as to recover the tritiated water quantitatively. The residue was taken up in 0.4 ml of water and the nucleotide was precipitated with 12 ml of redistilled acetone at -15°. After 45 minutes, the TPNT was centrifuged down, taken up with 10 ml of 0.01 M $NaHCO_3$, and placed on an 0.5 cm x 5.5 cm water-washed DEAE-cellulose column (72). The column was washed with 30 ml of 0.005 M potassium phosphate buffer, pH 7.2. Unreacted nucleotide was eluted with 10 ml of 0.10 M NaCl in the same buffer, and TPNT was eluted with 0.25 M NaCl in buffer. The yield of TPNT was 60-75%, and contained 19,000 cpm/μmole. (Although the principal purpose of the DEAE-cellulose column was to separate unreacted TPN^+ from TPNT, separate experiments showed that any $S_2O_4^=$ present would be eluted ahead of TPNH. In these separate experiments $S_2O_4^=$ was determined iodometrically (152)).

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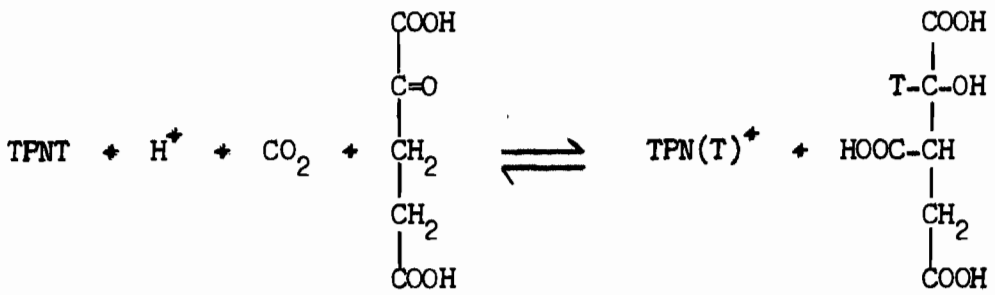
[5]Abbreviations: $TPN(T)^+$ and $DPN(T)^+$ represent oxidized forms of the nucleotides with tritium in the para position of the nicotinamide ring. TPNT and DPNT are the corresponding reduced nucleotides. The prefixes α- and β-, refer to the side of the nicotinamide ring to which tritium is attached, taking α-DPNT as the form which would lose its tritium atom to acetaldehyde in the alcohol dehydrogenase system (150). T_2O is tritiated water.



Glucose 6-phosphate and glucose 6-phosphate dehydrogenase were also present to recycle the $TPN(T)^+$ and shift the equilibrium towards isocitrate production (153). In a test tube were placed 2.0 ml of 2.0 M Tris acetate buffer, pH 7.5, 8.0 μmoles of $MnSO_4$, 6.0 μmoles of TPNT, 60 μmoles of α-ketoglutarate, 40 μmoles of glucose 6-phosphate, 4.0 ml of 0.10 M $NaHCO_3$ saturated with CO_2, and 400 units of TPN-specific isocitric dehydrogenase; final volume, 13.2 ml. The mixture was incubated for 45 minutes at 25°. Then 2.4 units of glucose 6-phosphate dehydrogenase were added, and the reaction allowed to continue 15 minutes more. The contents were cooled to 2°, and 7.0 ml of 2% (w/v) $NaHSO_3$ were added, followed in 5 minutes by 1.9 ml of concentrated H_2SO_4 and 14.4 gm of $MgSO_4.7H_2O$. The slightly turbid solution was extracted continuously with ether for 3 days according to Wood et al. (154). The ether layer was evaporated to dryness, taken up in 4.0 ml of water, and kept at pH 9.0 with KOH while heating at 90° for 10 minutes. After cooling, the solution was neutralized with HCl, diluted to 50 ml and applied to a water-washed 1.0 cm x 10 cm DEAE-cellulose column. Linear gradient elution was employed, with 100 ml of water in the mixing

chamber and 100 ml of 0.20 M NaCl in 0.005 M potassium phosphate buffer at pH 7.2 in the reservoir, initially. The purified tritiated isocitrate appeared between 80 and 100 ml of effluent. To concentrate the isocitrate, the pooled fractions were diluted and put onto an 0.3 x 3.0 cm DEAE-cellulose column. The isocitrate was then eluted with 0.3 M NaCl. Isocitrate was assayed enzymically with TPN-specific isocitric dehydrogenase, and it was found that 4.87 μmoles of isocitrate were recovered with 6250 cpm/μmole. When stored at pH 6.0 in the frozen state, isocitrate lost negligible amounts of radioactivity into water within 4 weeks.

α-Ketoglutarate was determined as the 2,4-dinitrophenylhydrazone according to the method of Koepsell and Sharpe (155). Lactate was assayed by a color reaction involving $CuSO_4$ and p-hydroxydiphenyl according to Barker and Summerson (156). Glutamate was determined by the ninhydrin method (61). Small amounts of DPN^+ (less than 0.1 μmole), were assayed by a modification of the method of LePage (157), which is suitable only for larger amounts of nucleotide. The modified method is as follows: The DPN^+ solution is made up to a final volume of 3.0 ml in a cuvette of 1.0 cm light path. 7.0 mg of $NaHCO_3$ are added and the solution mixed. After 6.0 mg of $Na_2S_2O_4$ are added, the solution is allowed to stand for 20 minutes. Then 18.8 mg each of Na_2CO_3 and $NaHCO_3$ are added. The solution is gently aerated for 15 minutes and the DPNH content determined from the optical density at 340 mμ.

Radioactivity was determined in a Packard scintillation counter[6] using the ternary system water:ethanol:toluene in the proportion 1:14:35. All counts were corrected for background. Counting time was at least 5 minutes, so that the standard error in counting was ± 3% or less.

RESULTS AND DISCUSSION

In designing the synthesis of isocitrate labeled with tritium in the α-position, it was preferable to avoid a chemical synthesis, since isocitric acid has two centers of asymmetry and four possible isomers. The naturally occurring isocitrate has the threo-D_s-configuration (11,12,13). Englard and Colowick's experiments (143) suggested that an enzymic synthesis of isocitrate with TPNT and the TPN-linked isocitric dehydrogenase was feasible, so that only threo-D_s-isocitrate-α-T would be produced. In the present experiments, the use of tritium as label, rather than deuterium as in most of Vennesland's studies on coenzyme stereospecificity, allowed the use of quite small amounts of material. The extent of the reductive carboxylation of α-ketoglutarate was enhanced by driving the reaction with the glucose 6-phosphate dehydrogenase system, which served to regenerate TPNH. Although the specific radioactivity of the isocitrate produced is diminished by production

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[6]The able instruction of Dr. Hans Rilling in the use of the scintillation counter is gratefully acknowledged.

of more unlabeled TPNH, the total label introduced into isocitrate should be increased for the following reasons: Since randomly labeled TPNT was employed in the enzymic synthesis, reduction of α-ketoglutarate leads to labeled isocitrate plus $TPN(T)^+$. When $TPN(T)^+$ is reduced by glucose-6-phosphate dehydrogenase, a β-specific enzyme (158), α-TPNT is produced. This in turn would give labeled isocitrate and unlabeled TPN^+ through the action of TPN-linked isocitric dehydrogenase, an enzyme with α-specificity (145). Thus, label from both sides of the randomly labeled nicotinamide ring of TPNT finds its way into isocitrate. Details of the synthesis are given under Materials and Methods.

Once threo-D_s-isocitrate-α-T had been produced, a direct transfer of the tritium to DPN^+ by DPN-specific isocitric dehydrogenase could be demonstrated. Experiment 1 of Table VII shows that the specific radioactivity of the original isocitrate and the DPNT isolated from DEAE-cellulose are about the same. Of the total radioactivity in isocitrate, the recovery in DPNT was about 75%, not inconsistent with the loss of DPNT during the chromatographic isolation and washing procedures. It, therefore, seemed unlikely that any extensive loss of tritium into the medium could have occurred. However, in this experiment the hydrogen acceptor, DPN^+ is present in excess; and it seemed possible that the enzyme might catalyze a relatively slow exchange of the tritium of isocitrate with water when DPN^+ was absent. In Table VIII are the results of experiments testing this possibility. In view of the

TABLE VII

TRANSFER OF LABEL FROM ISOCITRATE-α-T TO DPN$^+$

Experiment 1: Isocitrate-α-T + DPN$^+$ ⟶ DPNT + H$^+$ + α-ketoglutarate + CO_2

Compound	μmoles	total cpm	cpm/μmole
Initial isocitrate	2.00	12,500	6250
Isocitrate consumed	1.81	11,300[a]	6250
Recovered reduced DPN	1.45	8,440	5820

Experiment 2: DPNT + H$^+$ + pyruvate ⟶ labeled lactate + DPN$^+$

Compound	μmoles	total cpm	cpm/μmole
Initial DPNT (from experiment 1)	0.116	675	5820
Recovered lactate	0.107	630	5890
Recovered oxidized DPN	0.040	0	0

Experiment 3: DPNT + H$^+$ + α-ketoglutarate + NH_3 ⟶ glutamate + DPN(T)$^+$

Compound	μmoles	total cpm	cpm/μmole
Initial DPNT (from experiment 1)	0.109	635	5820
Recovered glutamate	0.087	12	140
Recovered oxidized DPN	0.096	538	5820

(continued)

TABLE VII, continued.

Experiment 1: The following mixture was incubated at 25° for 74 minutes: 0.05 ml of 2.0 M Tris acetate buffer, pH 7.5, 4.0 μmoles of $MnCl_2$, 2.03 μmoles of labeled isocitrate, 10.0 μmoles of DPN^+, 2.0 μmoles of ADP, and DPN-linked isocitric dehydrogenase (40 units); final volume, 3.0 ml. According to the change in optical density at 340 mμ, 89% of the isocitrate was oxidized in this time. The mixture was then heated at 90° for 1 minute, diluted with water to 50 ml, and passed through a 1 cm x 8 cm cellulose column which had been equilibrated with 0.005 M potassium phosphate buffer, pH 7.2. Linear gradient elution was produced with 150 ml of buffer in the mixing chamber and 150 ml of buffer plus 0.20 M NaCl in the reservoir. The order of elution was DPN^+, α-ketoglutarate, DPNH, and isocitrate. With a flow rate of 1.0 ml/minute, collections of 5 ml per tube yielded only sight overlap between DPNH and any isocitrate which was still unoxidized. The DPNH-containing fractions were pooled, diluted, and rechromatographed on an 0.5 cm x 5.5 cm DEAE-cellulose column using similar gradient conditions in order to concentrate the nucleotide and free it from the remaining isocitrate.

Experiment 2: An aliquot of DPNT produced in Experiment 1 was oxidized as follows: In a cuvette were placed 0.05 ml of 2.0 M Tris acetate buffer, pH 7.5, 0.80 μmole of sodium pyruvate, 0.12 μmole of DPNT, and 0.01 mg of lactic dehydrogenase; final volume, 3.0 ml. By observation at 340 mμ, it was determined that 0.118 μmole of DPNT was oxidized within 15 seconds. The solution was then heated for 1.5 minutes at 90° and cooled to room temperature. 7 mg of Norit A were added and the mixture stirred for 20 minutes. The charcoal was filtered off on a layer of Celite in a Buchner funnel. The Norit-Celite mixture was stirred with 3.0 ml of 10% isoamyl alcohol for 20 minutes (162). The filtrate of this mixture was extracted with ether and the aqueous layer taken to dryness under reduced pressure. The residue was taken up with water in a final volume of 6.5 ml. 3.0 ml was used for radioactivity determination, and another 3.0 ml was placed in a cuvette and assayed for DPN^+.

The filtrate left after Norit treatment was assayed for lactate and counted. The filtrate had no detectable DPN^+; conversely, control experiments showed no lactate in the eluate from Norit after ether extraction.

Experiment 3: Another aliquot of DPNT from Experiment 1 was oxidized as follows: in a cuvette were placed 0.05 ml of 2.0 M Tris acetate buffer, pH 7.5, 94 μmoles of α-ketoglutarate, 15 μmoles of NH_4Cl, 0.11 μmoles of DPNT, and 1.0 mg of glutamic dehydrogenase in ammonium sulfate suspension; final volume 3.0. After 15 minutes, essentially complete oxidation of DPNT had occurred. The separation of glutamate from DPN was accomplished as in experiment 2. After

TABLE VII, continued.

alkalinizing the filtrate with saturated K_2CO_3 ammonia was removed by aeration, and the glutamate determined. The filtrate contained less than 0.004 μmole of DPN^+. The DPN^+ was eluted from Norit with 10% isoamyl alcohol as in experiment 2, except that a contact time of 4 hours was used resulting in a much higher recovery of DPN^+.

[a]Calculated.

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fact that the very small number of counts in the water in experiments 1 and 3 are about the same as that obtained without enzyme, no enzymic labilization seems to occur. This was confirmed in experiment 4, where enzymic incorporation of tritium from T_2O into isocitrate could not be demonstrated.

Since TPN-linked isocitric dehydrogenase has been shown to be specific for the α-side of TPN^+ (145), it was of interest to investigate and compare the stereospecificity for DPN^+ of the DPN-linked enzyme. This was done by subjecting the DPNT recovered from experiment 1 of Table VII to oxidation by muscle lactic dehydrogenase and liver glutamic dehydrogenase (experiments 2 and 3 of Table VII). In experiment 2, excellent agreement was obtained between the specific radioactivities of the initial DPNT and the lactate which was produced by reduction of pyruvate, and the recovery of radioactivity in lactate was 94%. Since lactic dehydrogenase is α-specific (159), the fact that all the radioactivity appears in lactate rather than in DPN^+ shows that DPN-linked isocitric dehydrogenase must have α-specificity. This conclusion was confirmed by

TABLE VIII

STUDY OF THE EXCHANGE REACTION BETWEEN ISOCITRATE AND WATER

Experiment	Content of Incubation Mixture	Radioactivity[a]		
		Water recovered	Isocitrate recovered	Present initially in isocitrate
		cpm	cpm	cpm
1	Enzyme, Mn^{++}, labeled isocitrate	26	-	280
2	Same as 1, but no enzyme	33	-	280
3	Same as 1, plus DPNH	25	-	280
4	Enzyme, Mn^{++}, unlabeled isocitrate, T_2O	4.0×10^9[b]	0	-

Experiment 1: The following mixture was incubated at 25° for 60 minutes: 0.05 ml of 2.0 M Tris acetate buffer, pH 7.5, 4.0 µmoles of $MnCl_2$, 0.2 µmoles of tritiated isocitrate with 1400 cpm/µmole, 0.2 µmole of ADP and 40 units of DPN-specific isocitric dehydrogenase. Final volume, 0.34 ml. Afterwards, the reaction mixture was lyophilized, and the water counted. The residue could not be counted directly because the large amount of solutes resulted in phase separation in the counting vials.

Experiment 2: Same as experiment 1 except that no DPN-specific isocitric dehydrogenase was added; final volume was 0.29 ml.

Experiment 3: Same as experiment 1 but with 0.1 µmole of DPNH added; final volume was 0.36 ml.

Experiment 4: The following mixture was lyophilized: 0.05 ml of 2.0 M Tris acetate buffer pH 7.5, 0.02 ml of 0.2 M $MnCl_2$, 0.2 ml of unlabeled threo-D_sL_s-isocitrate and 40 units of DPN-linked isocitric dehydrogenase. To the residue was added 0.196 gm of T_2O with a specific radioactivity of 100 mc/ml. The solution was left at 25° for 2 hours and lyophilized. Enzyme acticity was destroyed with perchloric acid. The isocitrate was re-isolated and washed on DEAE-cellulose as described under Materials and Methods.

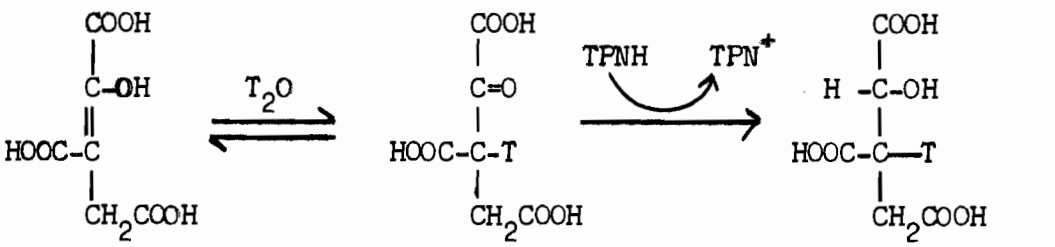
[a]Total counts added or recovered per experiment.

[b]Added.

utilizing the glutamic dehydrogenase reaction, which is β-specific (160). In experiment 3, the specific radioactivity of the DPN(T)$^+$ recovered was in good agreement with that of the DPNT initially present, and the yield of radioactivity in the oxidized nucleotide was 85%. This indicates that the hydrogen put onto DPN$^+$ by DPN-specific isocitric dehydrogenase is not removed by glutamic dehydrogenase.

These results show that DPN-linked isocitric dehydrogenase from bovine heart catalyzes the removal of the α-hydrogen atom of isocitrate and transfers it directly to the α-side of the nicotinamide ring of DPN$^+$. Except for the difference in coenzymes, this is also what occurs in the case of TPN-linked isocitric dehydrogenase from the same source.

Direct evidence has been obtained that the β-hydrogen of isocitrate is not lost in <u>either</u> isocitric dehydrogenase reaction. <u>Threo-D$_s$</u>-isocitrate-β-T has been synthesized by reduction of oxalosuccinate in T_2O with TPNH and TPN-linked isocitric dehydrogenase.



oxalosuccinate (enol form) | oxalosuccinate (keto form) | <u>Threo-D$_s$</u>-isocitrate

On oxidation of this isocitrate with either the DPN- or the TPN-isocitric dehydrogenase, α-ketoglutarate was obtained which contained essentially all of the radioactivity, while the reduced pyridine nucleotides formed were unlabeled (Table IX). The demonstration of the transfer of label from the β-hydrogen of isocitrate to α-ketoglutarate by TPN-linked isocitric dehydrogenase would give direct support to the observations of Rose et al. (161) who had oxidized citrate-α-T in the presence of aconitase and a large amount of TPN-specific isocitric dehydrogenase to α-ketoglutarate containing about 30% of the label. Presumably, aconitase formed isocitrate-β-T, which was then oxidized to α-ketoglutarate without loss of label. The label which was lost, apparently went into the medium during the aconitase reaction. Direct evidence has been provided in the present experiments that the β-hydrogen of isocitrate is retained in α-ketoglutarate in either isocitric dehydrogenase reaction. Hence, the enol form of oxalosuccinate is not likely to be an intermediate in the oxidation of isocitrate by either enzyme.

Separate experiments were designed to test the possibility that DPN-linked isocitric dehydrogenase might actually produce oxalosuccinate rather than α-ketoglutarate from isocitrate. Plaut and Sung (5) had oxidized isocitrate in the DPN-specific system, stopped the reaction by boiling, and isolated α-ketoglutarate as the 2,4-dinitrophenylhydrazone on silica gel columns. It seemed possible that any oxalosuccinate which was present would

TABLE IX

OXIDATION OF THREO-D_s-ISOCITRATE-β-T

Substance	Isocitric dehydrogenase reaction	μmoles	Total cpm	cpm/μmole
Initial isocitrate-β-T[a]	DPN or TPN	0.111	711	6460
Recovered α-ketoglutarate	DPN	0.084	418	4980
Recovered reduced DPN	DPN	0.052	0	0
Recovered α-ketoglutarate	TPN	0.097	438	4520
Recovered reduced TPN	TPN	0.078	8	100

To 0.30 ml of T_2O (50 mc/ml) was added 0.20 ml of 0.016 M oxalosuccinate, pH 7.0. After 10 minutes at 25°, the following were added: 0.015 ml of 2.0 M imidazole buffer, pH 5.8; 0.03 ml of 0.02 M $MnCl_2$; 0.05 ml of 0.005 M TPNH; and 0.01 ml of TPN-linked isocitric dehydrogenase solution containing 2 units. After standing for 2.5 hours, the reaction mixture was lyophilized. To the residue were added 0.5 ml of 1.0 M H_2SO_4 and 1.0 ml of 0.001 M $Al_2(SO_4)_3$, followed by 50 ml of water and 8 μmoles of threo-D_sL_s-isocitrate carrier. Labeled isocitrate was obtained by resolution on DEAE-cellulose as described under Materials and Methods.

The labeled isocitrate was oxidized in both the DPN- and TPN-specific isocitric dehydrogenase systems, and the pyridine nucleotides were separated by Norit treatment as in Table VII. The filtrates were extracted with ether. The residues after evaporation of the ether were taken up in a small amount of water for radioactivity determination. The prolonged ether extraction probably resulted in some loss of label from α-ketoglutarate into water by enolization.

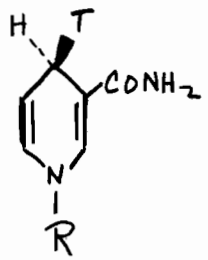
[a]Determined as threo-D_s-isocitrate with TPN-linked isocitric dehydrogenase.

be decarboxylated to α-ketoglutarate by heating in the presence of metal ions. Therefore, attempts were made to identify oxalosuccinate by addition of 2,4-dinitrophenylhydrazine reagent without prior boiling of the reaction mixture. When chromatographed on silica gel columns with the chloroform: n-butanol system of Brummond and Burris (149), the product of the oxidation of isocitrate appeared to migrate with the R.F. of α-ketoglutarate rather than of oxalosuccinate. These experiments merely confirmed, then, that the DPN-linked isocitric dehydrogenase reaction is a concerted oxidative decarboxylation.

IV. COMMENTS

The results of this study show that the hydrogen transfer properties of the two isocitric dehydrogenases are quite similar. Experiments which employ TPN-linked isocitric dehydrogenase for studies on the stereochemistry of the Krebs cycle hydrogen transfers (for review, see Englard (163)) tacitly imply that it is TPN-linked enzyme which is chiefly responsible for isocitrate oxidation in the cycle. However, such experiments would have yielded the same results if the DPN-linked isocitric dehydrogenase had been used. If the two isocitric dehydrogenases had transferred different hydrogens of isocitrate to their respective coenzymes, it might have been possible to have designed experiments to determine which enzyme was the Krebs cycle enzyme on the basis of loss or retention of label in various citric acid cycle intermediates.

Recently, Cornforth et al. (164) have determined the absolute configuration of the DPND produced by alcohol dehydrogenase and deuterated substrate. Since DPN-linked isocitric dehydrogenase has now been shown to have the same nucleotide stereospecificity as alcohol dehydrogenase, we may write the following structure for the α-DPNT resulting from oxidation of isocitrate-α-T:



R = ADP-ribosyl-

Since oxalosuccinate has been shown not to accumulate in the TPN-linked isocitric dehydrogenase system of pig heart (19), and since the present studies show that oxalosuccinate does not appear in the DPN-linked system, no pathway is known in mammalian systems whereby oxalosuccinate can be produced. The situation is reminiscent of the finding by Speyer and Dickman (165) that cis-aconitic acid was not an obligatory intermediate in the aconitase reaction. However, it seems to be established that cis-aconitate can arise as the result of aconitase; and indeed, the concentrations of cis-aconitate in certain cells has been measured (166). Since it has been claimed that oxalosuccinate occurs in plant mitochondria (149), there may be an as yet unknown pathway for the synthesis of this compound.

CHAPTER V

STUDIES ON THE MECHANISM OF ACTIVATION BY ADP

The studies of Chapters II and III indicate that DPN-linked isocitric dehydrogenase of bovine heart is profoundly influenced by ADP. The following are expressions of the effect of ADP: 1. K_m for isocitrate is decreased markedly while V_{max} is unchanged. 2. K_m for metal ion is also decreased markedly, while V_{max} is unchanged. 3. The pH optimum of the enzyme is shifted to pH 7.2. 4. The enzyme is stabilized by ADP at low ionic strength. In considering the possible mechanism by which ADP causes these effects, one is attracted to recent work done on glutamic dehydrogenase of beef liver, an enzyme which is in several ways similar to DPN-linked isocitric dehydrogenase. Both enzymes are exclusively mitochondrial (5,167), and the two enzymes are the only examples of enzymes which are stimulated by ADP. In addition, glutamic dehydrogenase seems sensitive to a number of other nucleotides, being stimulated by 5'-AMP and inhibited by ATP, GTP, and high concentrations of DPNH (89,108). Both enzymes thus seem surprisingly responsive to a variety of nucleotides. The similarities between the enzymes suggested that certain findings concerning the mechanism of ADP stimulation of glutamic dehydrogenase might be applicable to DPN-linked isocitric dehydrogenase.

Olson and Anfinsen (139) reported that glutamic dehydrogenase had a molecular weight of 1×10^6 as determined by sedimentation and diffusion studies. The sedimentation constant of 26.6 S was calculated by extrapolation to zero protein concentration after making a series of measurements on solutions of various concentrations. It was noted that the molecule dissociated into subunits if the protein concentration in the ultracentrifuge cell was below 2.5 mg per ml (0.25%). In kinetic studies, Frieden (168) found that concentrations of DPNH above 4×10^{-4} M caused inhibition of glutamic dehydrogenase and also caused the enzyme to dissociate, even at concentrations which normally favored aggregation of subunits to form the molecule of 1×10^6 molecular weight. The sedimentation velocities suggested that the molecule dissociated into four subunits, each with a molecular weight of 250,000. The correlation between dissociation by DPNH and DPNH inhibition seemed excellent, so it was, therefore, assumed that the subunits were inactive and only the protein with molecular weight of 1,000,000 was active. This assumption was strengthened by the finding that ADP both stimulated glutamic dehydrogenase and favored its aggregation. ADP prevented the dissociation caused by DPNH (89). Yielding and Tomkins (107) subsequently found that certain aromatic compounds including estrogens and the non-steroidal estrogen analogue, diethylstilbestrol, both inhibit and cause dissociation of the molecule. Wolff (169) found that thyroxine and triiodothyronine also would dissociate glutamic dehydrogenase and inhibit.

Thus, it appeared that correlation between activity and association, or, conversely, inhibition and dissociation, was extremely good.

However, when one looks at the data of Olson and Anfinsen (139), it is apparent that enzyme would probably be completely dissociated at the concentrations used in enzymic assays where only a few micrograms of protein are present. It is, therefore, not easy to understand why it has always been assumed that the true molecular weight of glutamic dehydrogenase is 1,000,000 rather than 250,000. Fisher et al. (109), in fact, have recently proved that the subunit is active. These authors performed enzyme assays using protein concentrations as high as used in ultracentrifugal analyses. In order to show the reaction velocity to a measurable rate, they employed an inhibitor, glutaric acid, which had previously been shown to have no effect on the association-dissociation properties of the enzyme. Exact correlation between the kinetic and ultracentrifugal data revealed that the enzyme was active as the monomer. Frieden (108) has now confirmed these results. By extrapolating light scattering data to low concentrations of protein, he has come to the conclusion that the enzyme is the "subunit" form in the concentrations used for enzyme assay.

It is evident that there must then be two species of subunits of glutamic dehydrogenase, one active, and one inactive, the latter being produced by inhibitors such as DPNH and diethylstilbestrol. The phenomenon of aggregation which is promoted by ADP can only be taken as an indication that the protein configuration is altered

in some manner so as to favor association. However, it is not certain whether at the low concentrations used for kinetic studies, ADP-stimulated enzyme is in the aggregated form.

Since ADP stimulated DPN-linked isocitric dehydrogenase, it was thought possible that a similar association-dissociation phenomenon might be demonstrable. Indeed, one of the main reasons for developing the purification techniques and obtaining relatively large quantities of pure enzyme was to perform ultracentrifugal studies in the presence and absence of ADP. In addition to these experiments, it seemed desirable to demonstrate that ADP itself was binding to the enzyme rather than AMP or phosphate. In other words, the enzyme might react with ADP in either of the following ways to form an activated enzyme complex.

1. Enzyme + ADP $\longrightarrow$ Enzyme-AMP + P_i
2. Enzyme + ADP $\longrightarrow$ Enzyme-P + AMP

This chapter presents certain data related to these problems. The results obtained suggest that ADP binds to the protein and causes an aggregation of DPN-linked isocitric dehydrogenase such as is noted with the subunits of glutamic dehydrogenase.

MATERIALS AND METHODS

Sephadex G-100 was obtained from Pharmacia Fine Chemicals, Inc., Rochester, Minn. The fines were removed and the gel thoroughly equilibrated with 0.10 M potassium phosphate buffer, pH 7.2,

before use.

ADP^{32} was prepared by Dr. G. W. E. Plaut (55). The radioactive nucleotide was isolated by paper chromatography in the system of Krebs and Hems (63). The nucleotide was eluted from paper with water and further purified by chromatography on DEAE-cellulose, which was found to be capable of separating 5'-AMP, ADP, and ATP. This method of purification appeared more convenient than chromatography on Dowex 1, a procedure which requires the use of formate for elution (170). The procedure developed for purification of adenine nucleotides is as follows:

A. Separation of Nucleotides. The following operations are performed in the cold room.

A 1.0 x 6.0 cm DEAE-cellulose column is packed using an aquarium pump, so that a flow rate of 0.8 ml/min is obtained with a pressure of 20 cm of water. The column is then washed with 200 ml of water. The sample containing adenine nucleotides is diluted until the ionic strength is below 0.01 and then is applied to the column. Gradient elution is then carried out using 150 ml of H_2O in the mixing chamber and 150 ml of 0.25 M NaCl in 0.005 M potassium phosphate, pH 7.2, in the reservoir. The flow rate is regulated by a Mini-pump at 1.5 ml/min. Fractions are collected every 5 min (7.5 ml/fraction). The column handles at least 15 µmoles of nucleotides and is useful in the 1-2 µmole range since recovery is very nearly quantitative.) The order of elution is as follows: 5'-AMP (approximately 115-142 ml of effluent), ADP (approximately

162-210 ml), and ATP (about 202-250 ml). The slight overlap between ADP and ATP can probably be avoided by carrying out the column separation at a higher pH.

B. Concentration of the Desired Fraction. The following operations can be done at room temperature since only a short time is required. The desired fractions, e.g., those containing ADP, are pooled and diluted with an equal volume of water. An 0.5 x 5.0 cm DEAE-cellulose column is prepared as above and washed with 100 ml of water. The diluted pool containing the desired nucleotide is applied to the column, which is then washed with 10 ml of water. The nucleotide is then eluted by applying four successive 3.0 ml portions of 0.25 M NaCl in 0.005 M potassium phosphate, pH 7.2. The nucleotide usually will all be in the second fraction.

All other reagents used in the studies of this Chapter have been previously described.

Radioactivity was determined by plating samples in planchets and counting with a thin window gas flow Geiger counter (Nuclear-Chicago, Model CB-110B).

Sedimentation experiments were performed by Mr. D. M. Brown with a Spinco Model E ultracentrifuge.

II. RESULTS

Binding studies. An attempt was made to identify a complex between the enzyme and its activator, ADP. In these experiments, mixtures of ADP^{32} and enzyme were passed through a Sephadex column.

If radioactivity appeared in the protein peak, this would indicate binding of P^{32} to the protein. The experiments would show only that ADP^{32} or P^{32} was attached to the protein, but really would not exclude the possibility that such binding was non-specific.

In the experiment shown in Fig. 16, radioactivity is clearly shown to be carried into the protein peak. The protein used for this experiment was not homogeneous in the ultracentrifuge, but a major component comprised about 80% of the protein present. Therefore, the radioactivity is probably associated with the enzyme itself rather than to other components. Since the specific radioactivity of the ADP^{32} was known, it was possible to calculate a molar ratio of ADP^{32} to enzyme, based on a specific activity of 5520 units per mg for the pure enzyme. The three most active fractions containing enzyme in the experiment of Fig. 16 had P^{32}/enzyme ratios of 2.3, 3.5, and 6.6. The variation in molar ratios of radioactivity to enzyme activity suggested that the small column, 1.0 x 17 cm, had not completely separated uncombined ADP^{32} from the protein peak.

In another experiment, a longer Sephadex G-100 column, 0.8 x 48 cm, was employed. The results are given in Fig. 17. Again, radioactivity due to P^{32} was found in the protein peak. However, calculations showed that the molar ratio of P^{32} to enzyme was between 1/30 and 1/18 for the enzymically active fractions of Fig. 17. It, thus, appears that the binding of P^{32} to protein occurs, but it is quite loose, so that prolonged passage through

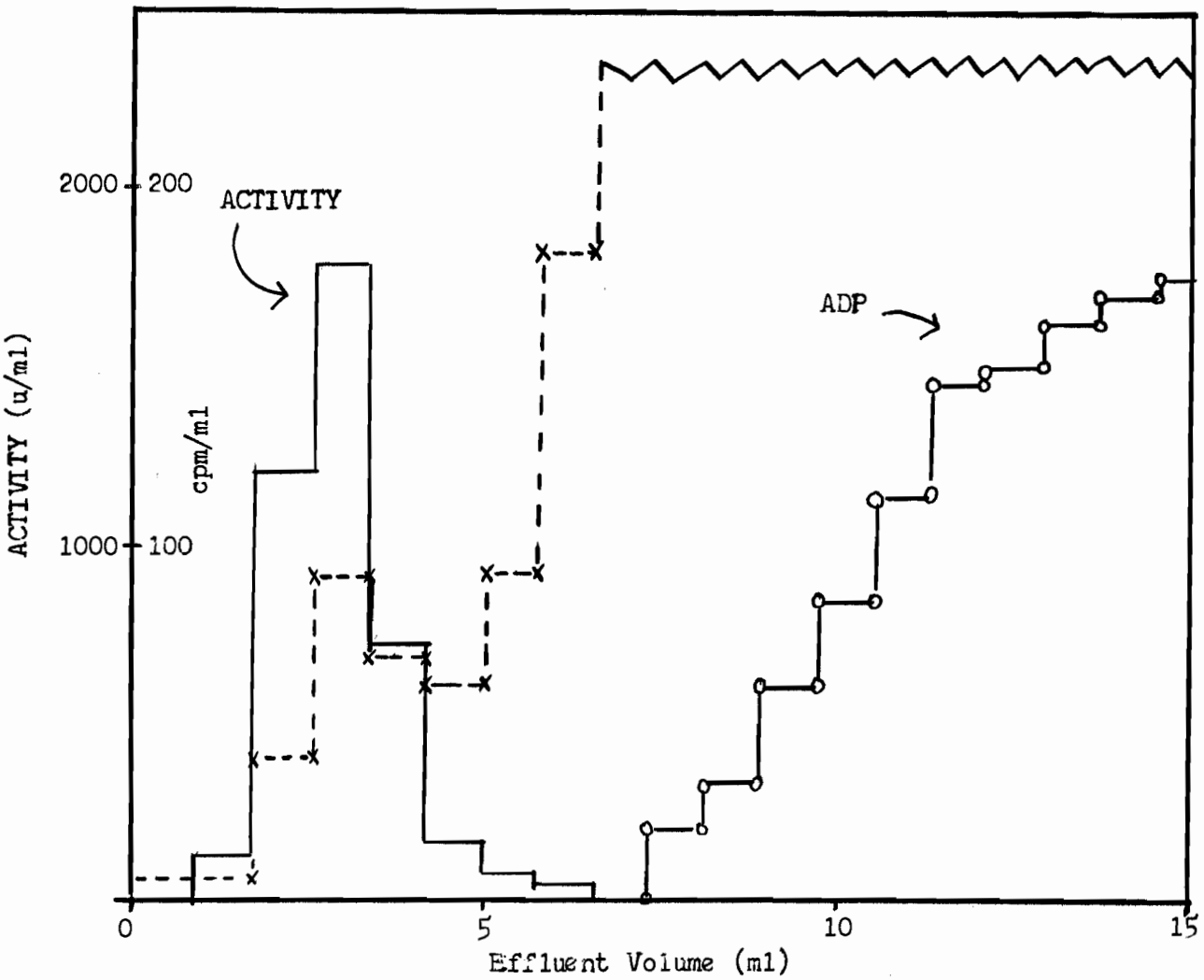


Fig. 16. Separation of a solution containing ADP^{32} and enzyme on Sephadex G-100.

A solution of DPN-linked isocitric dehydrogenase was treated with ammonium sulfate to precipitate the enzyme at 0.5 saturation. The precipitate was taken up in 1.0 ml of 0.0064 M ADP^{32}. The resulting enzyme solution had a specific activity of 3000 units/mg and contained 1.0 mg of protein. The solution was then placed on a Sephadex G-100 column (1.0 x 17 cm) previously equilibrated with 0.10 M potassium phosphate buffer solution, pH 7.2. The column was then washed with the same buffer and fractions of about 0.8 ml collected. The volume of each fraction was determined gravimetrically, assuming a specific gravity of 1.0 gm/ml. The radioactivity of the fractions was determined and the enzymic activities determined. ADP content was measured by the absorption at 260 mμ. The portions of the elution pattern representing ADP and enzymic activity are represented by solid lines, whereas the dotted lines indicate radioactivity. The specific radioactivity of the ADP^{32} was 230,000 cpm/μmole.

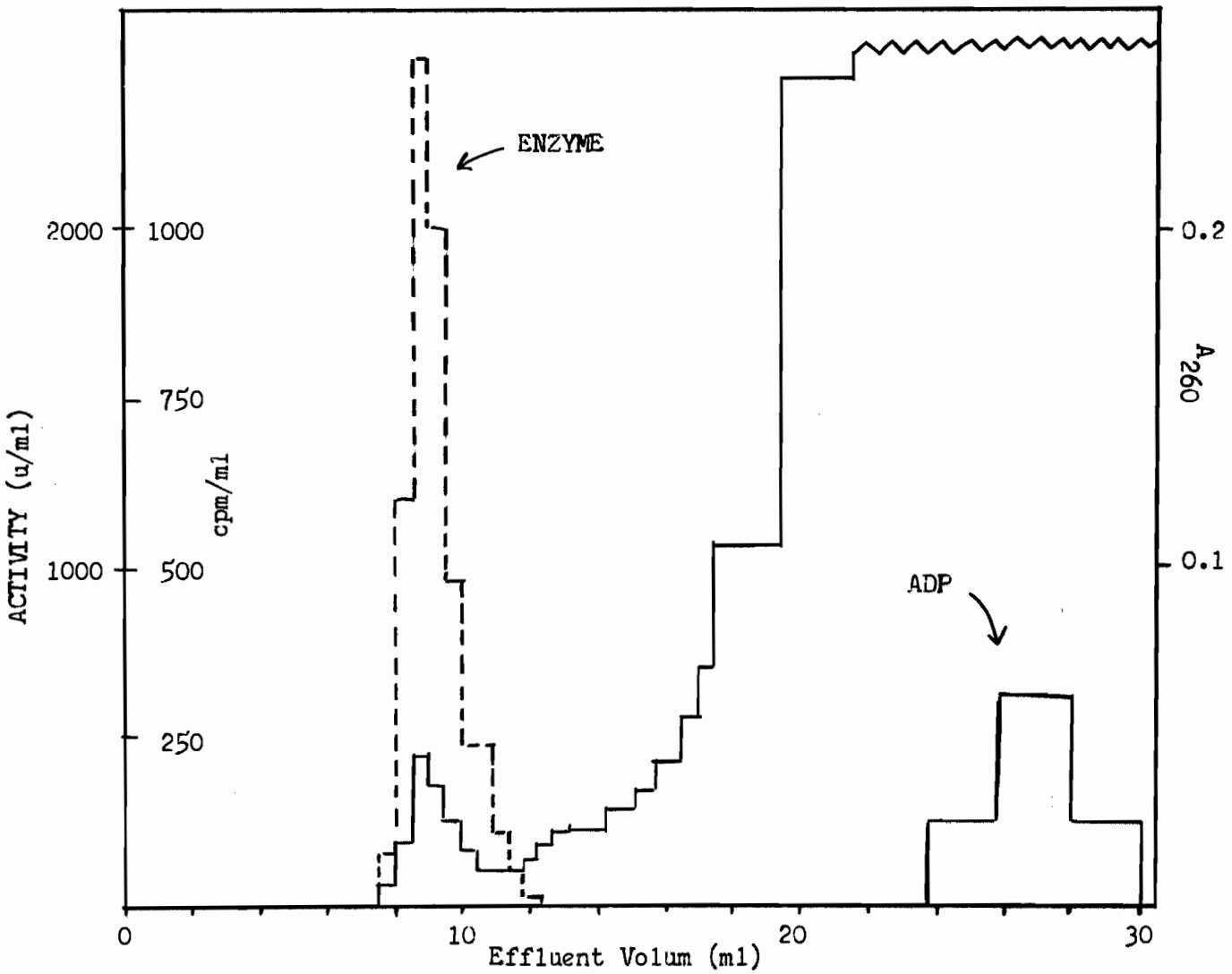


Fig. 17. Removal of enzyme-bound radioactivity by chromatography on Sephadex G-100.

1.0 mg of DPN-specific isocitric dehydrogenase having a specific activity of 3000 was dissolved in 0.8 ml of 0.10 M potassium phosphate buffer, pH 7.2, containing 0.24 μmoles of ADP^{32} with a specific radioactivity of 4.4×10^6 cpm/μmole. The mixture was chromatographed on an 0.8 x 48 cm Sephadex G-100 column as in Fig. 16. The dashed line represents the enzymic activity. Radioactivity is given by the solid lines, as is ADP, which could be distinguished by absorption at 260 mμ in later fractions but was probably present in too low a concentration in earlier fractions to be seen spectrophotometrically.

a Sephadex column results in dissociation of the complex. The results suggested that the radioactive protein consisted of an enzyme-ADP^{32} complex rather than a phosphorylated enzyme, since the latter would involve covalent bonding, which might be expected to be stronger than the binding shown in these experiments.

The looseness of binding confirms the impression given by certain facts already brought out in Chapter II; namely, the rather high ADP concentration of about 4×10^{-4} M required for half-maximal stimulation (Fig. 5), and the fact that the enzyme, as isolated from acetone powders, apparently contains no bound ADP, since enzyme at all stages of purification is stimulated by ADP to about the same extent.

Sedimentation experiments. It was shown in Chapter III that highly purified preparations of DPN-linked isocitric dehydrogenase show a major component with a sedimentation rate of 10.3 S (Fig. 15). A number of experiments have shown that ADP altered this sedimentation pattern. Fig. 18 shows the effect of adding 6.7×10^{-4} M ADP to enzyme which had previously shown only a major peak at 10.3 S and a minor component (6.2 S). This concentration of ADP caused almost complete disappearance of the 10.3 S peak, while a heavy component appears (21.8 S). The appearance of a heavy component indicates aggregation of the protein, but without diffusion studies, it is not possible to say how many units are present in each aggregate. It seems possible that the ADP-aggregated molecule may be a trimer or tetramer of the original

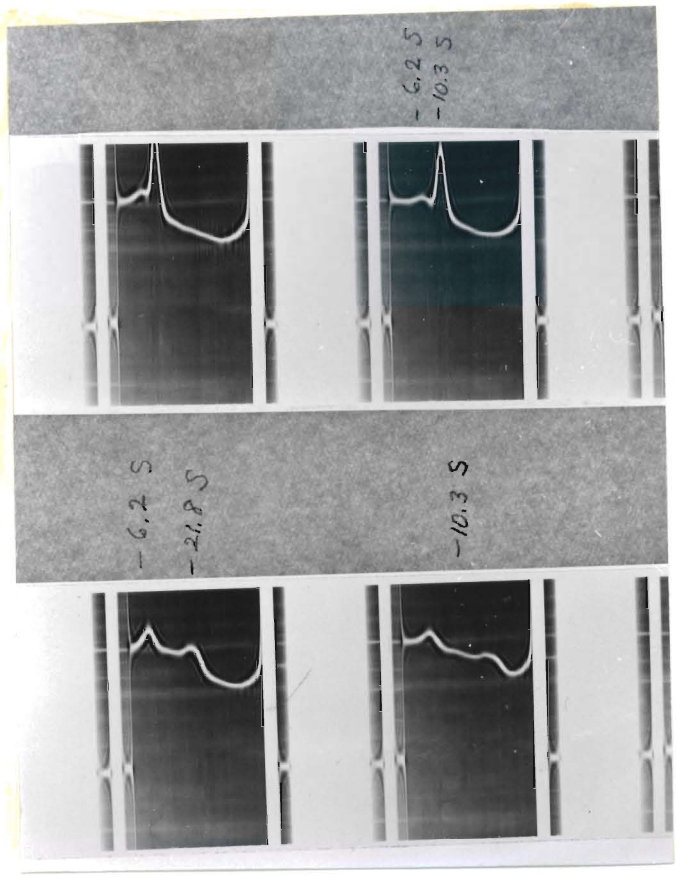


Fig. 18. The effect of ADP on the sedimentation pattern in the ultracentrifuge.

The schlieren patterns of two ultracentrifugal analyses are shown. In both cases the enzyme had been dialyzed thoroughly against 0.10 M potassium phosphate buffer, pH 7.2. Sedimentation is from left to right. Pictures were taken at 24 and 32 minutes after reaching a speed of 59,780 rpm. Temperature, 20°.

0.8 ml out of approximately 1.2 ml of protein solution (10 mg/ml, specific activity, 3200) was centrifuged in the experiment shown at the top of the figure. After ultracentrifugation the protein solution was recovered and found to have developed a precipitate. The recovered solution was mixed with that remaining solution which had not been ultracentrifuged, and all insoluble material was removed by centrifugation. After the protein in the supernatant was precipitated by ammonium sulfate treatment, it was collected by centrifugation, dialyzed against 0.10 M potassium phosphate buffer, pH 7.2, and sufficient ADP was added so that a final concentration of 6.7×10^{-4} M was obtained. The specific enzyme activity was 2000 units per mg. The sedimentation of this solution appears in the bottom row.

material sedimenting with a constant of 10.3 S. In Fig. 18, there is a suggestion of a small component still sedimenting in the range of 11 S in the presence of 6.7×10^{-4} M ADP. Possibly this concentration of ADP is almost high enough to cause complete aggregation of the enzyme, but smaller concentrations of ADP give no change in sedimentation rate, as shown in the experiment of Fig. 15 of Chapter III, in which the major component sedimented at 10.3 S after equilibration with 1×10^{-5} M ADP.

Because DPNH inhibited the enzyme (Chapter II), it was expected that this nucleotide might have a different effect than ADP on the sedimentation rate of the enzyme. In Fig. 19 is shown the sedimentation pattern obtained in the presence of DPNH and in the presence of DPNH plus ADP. It is obvious that DPNH produces no aggregation of enzyme, which appears as a 10.3 S component. However, Fig. 19 also shows that the presence of DPNH prevents aggregation of the protein by ADP. In the presence of both nucleotides, the sedimentation coefficient of the major component was calculated to be 11.0 S. Although aggregation has apparently been prevented, the sedimentation pattern is quite different. In this experiment, the presence of both DPNH and ADP seemed to broaden the major peak and cause an increase in size of the minor component. It is possible that the major component may represent protein which is binding both ADP and DPNH. It will be remembered that in Chapter II, it was shown that DPNH inhibits the enzyme, while ADP activates, but does not reverse the inhibition by DPNH in a competitive manner.

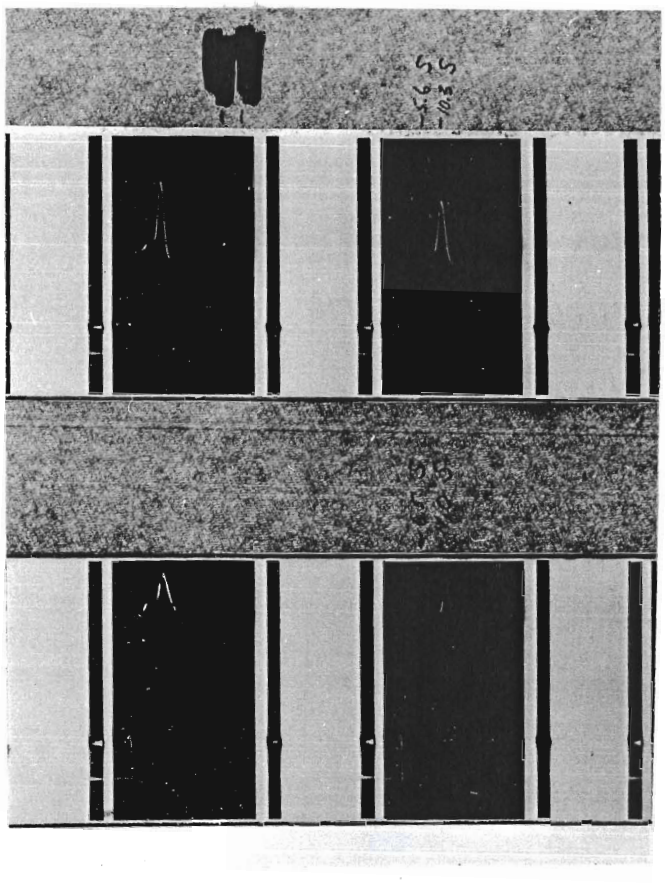


Fig. 19. The effect of DPNH and of DPNH plus ADP on the sedimentation pattern.

The conditions for analysis were the same as in Fig. 18, but the pictures shown were taken at 16 and 24 minutes after reaching 59,780 rpm. A single sample of enzyme was divided in half after dialysis for the two analyses.

TOP: Enzyme specific activity, 2,220. DPNH, 3.2×10^{-4} M, present.

BOTTOM: Another aliquot of the above enzyme preparation contained 3.2×10^{-4} M DPNH for two hours at 2° before ADP was added (final concentration, 6.7×10^{-4} M) and ultracentrifugation was performed.

Although aggregation of DPN-linked isocitric dehydrogenase seems to occur, it is not known if the enzyme consists of yet smaller subunits. In all ultracentrifugal experiments so far performed, components with sedimentation coefficients less than 10.3 S have always been noted. These components have ranged from 4.6 to 6.5 S. Although these components probably are impurities, there is the possibility that they may represent smaller subunits of the 10.3 S molecule. For instance, in Fig. 19 (bottom) the amount of minor component appears to have increased with time compared to the experiment shown in the top row of Fig. 19, although the same preparation of protein was used in each run.

Reversibility of the Aggregation. It was found that the aggregation by ADP could be reversed by dialysis. Following the experiment of Fig. 18 (bottom) in which the presence of ADP caused the appearance of a 21.8 S component, the enzyme was recovered from the cell compartment. The specific activity of the enzyme was found to have fallen to 1950 units per mg. The protein was combined with another preparation of enzyme made by the "Alternate Procedure" of Chapter III and precipitated by ammonium sulfate treatment. The protein was collected and dialyzed as before against 0.10 M potassium phosphate buffer, pH 7.2. Ultracentrifugal analysis of the protein showed a major component sedimenting at 10.4 S and containing no suggestion of any component sedimenting in the 21 S range.

A similar experiment performed with a sample of enzyme in the

presence of 1×10^{-3} M ADP showed a major component with a sedimentation constant of 20.4 S. After recovery of protein from the cell, precipitation of protein with ammonium sulfate, and dialysis against 0.10 M potassium phosphate buffer, pH 7.2, the solution contained a major component with a sedimentation coefficient of 10.3 S, and no heavier components.

III. DISCUSSION

The results of the binding experiments suggest that ADP binds to the enzyme, although the complexes Enzyme-P or Enzyme-AMP are not strictly ruled out. It seems more likely, however, that the activated complex consists merely of enzyme and ADP, because covalent bonding which might occur would probably be evidenced by stronger binding phenomena than have been noted. Thus, enzyme can be exposed to ADP, dialyzed, and then exhibit the same degree of stimulation by ADP as originally found. The aggregation produced by ADP also seems to be completely reversible by dialysis.

The binding experiments are similar to those performed by Martin (171) who used Sephadex G-25 columns to separate radioactive ATP and histidine from a ternary enzyme complex formed by these compounds and the enzyme, phosphoribosyl-ATP pyrophosphorylase, which is involved in histidine biosynthesis. Martin (171) was able to demonstrate a stoichiometric relationship between enzyme and both ATP and histidine in the enzyme-complex. However, only

small Sephadex columns were used (0.8 x 8 cm). Since the enzyme-complex should be in equilibrium with the smaller molecules, it might be expected that longer Sephadex columns would tend to dissociate the complex. In fact, if one knew the column volume, void volume, and the amount of complex obtainable with columns of various sizes, one should have enough information to derive the dissociation constant of the complex. According to Martin (171), a theoretical analysis of the Sephadex technique of studying complexes is now being prepared.

In the case of DPN-linked isocitric dehydrogenase, the Sephadex column studies show that ADP can be removed from the protein if the column is large enough. Isolation of an enzyme-complex would be favored in those instances where a covalent bond is formed. An example is the case of the complex between acetyl-CoA carboxylase and $C^{14}O_2$, which was isolated by Waite and Wakil (172), who used Sephadex G-25 to retard uncombined $C^{14}O_2$.

The ultracentrifugal analyses indicate that DPN-linked isocitric dehydrogenase is aggregated by ADP and kept in an unaggregated form by DPNH. The results are similar to those obtained by Frieden (89) for glutamic dehydrogenase. One difference which is immediately obvious is that the DPN-linked isocitric dehydrogenase apparently has little propensity for aggregation in the absence of ADP, whereas glutamic dehydrogenase in concentrations above 0.25% tends to be in a tetrameric form. Both enzymes, however, in the very minute concentrations which are employed for kinetic and assay

experiments, are probably in the unaggregated form. The aggregation phenomenon is a clear demonstration of a conformation change in the enzyme, but does not mean that the enzyme is aggregated either *in vivo* or in most reactions run *in vitro*.

Aggregation by an activating compound is not limited to glutamic dehydrogenase and DPN-linked isocitric dehydrogenase. Some evidence has been obtained by Vagelos *et al.* (173) with adipose tissue acetyl-CoA carboxylase that citrate stimulation is accompanied by a conformational change in the enzyme protein. These workers find that the enzyme sediments faster in a sucrose gradient in the presence of citrate, and suggest that this is due to aggregation of the enzyme. Noltmann and Kuby (174) have reported that TPN^+ causes an aggregation of yeast glucose 6-phosphate dehydrogenase (probably a dimerization). The same enzyme from human erythrocytes has recently also been found to sediment more rapidly in the presence of TPN^+ (175,176). It has been shown that glucose 6-phosphate of red cells is stabilized and activated by TPN^+ (175).

It should be noted that although DPN-linked isocitric dehydrogenase requires metal ions for activity, aggregation was demonstrated in these studies with ADP in the absence of added Mn^{++} or Mg^{++}. It seems probable, therefore, that ADP itself binds to the protein rather than as an ADP-metal complex. The presence of enzyme-bound metal has, however, not been eliminated.

If the conformational change induced by ADP is directly related

to the activation effect, one may picture the interaction of ADP and enzyme in the manner depicted in Fig. 20. In this schematic diagram, the enzyme is shown with a group, X, which partially blocks the site for binding of the isocitrate-metal ion complex. The group X may be displaced by increasing the amount of isocitrate so that when complete displacement is achieved, V_{max} is obtained. ADP is capable of permitting V_{max} to be reached at a much lower concentration of isocitrate because the nucleotide causes a conformational change which completely removes group X from the site of isocitrate binding. Although this scheme is speculative, it is consistent with the fact that V_{max} is the same with and without ADP. In fact, the Lineweaver-Burk curves for velocity as a function of isocitrate or metal ion concentration in the presence and absence of ADP are of the form found in cases of strictly competitive inhibition (Figures 6 and 7). Stimulation by ADP can then be considered as removal of an intramolecular completitive inhibitor.

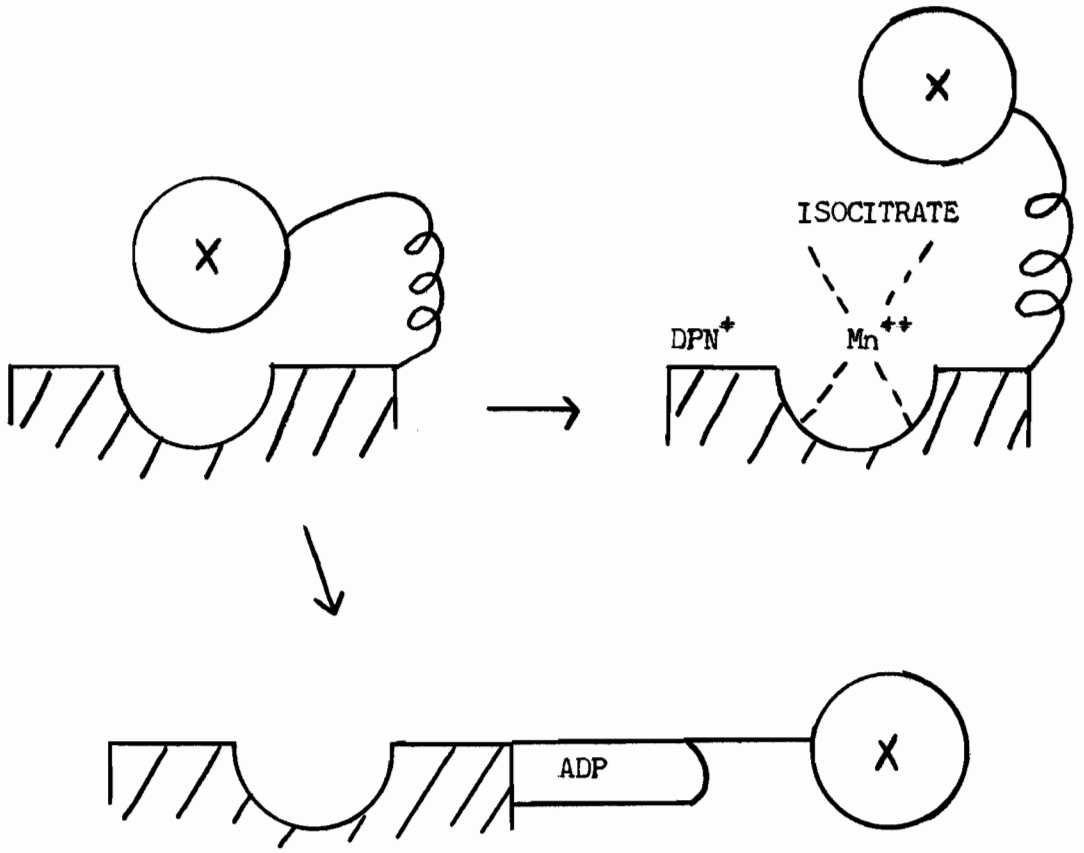


Fig. 20. Hypothetical picture of the catalytic site of DPN-linked isocitric dehydrogenase.

Note that the binding site for DPN^+ must be different from that for isocitrate. ADP is shown altering the conformation of the protein so that group X is removed from the area of the substrate binding site.

CHAPTER VI

DISCUSSION

These studies of DPN-linked isocitric dehydrogenase from bovine heart mitochondria havebeen fruitful in a number of areas. Possibly the most interesting of the findings has been the fact that the enzyme is activated and inhibited by certain nucleotides. In terms of the significance which this finding may have with regard to cellular metabolism, it should be recalled that mitochondria are the site of oxidative phosphorylation, a process which involves the very same nucleotides which affect DPN-linked isocitric dehydrogenase. Since this enzyme may be an intimate part of the Krebs cycle, a pathway for the oxidation of many substrates, the enzyme may play a pivotal role in the regulation of oxidation of certain substrates in accord with the energy needs of the cell.

Fig. 21 depicts the electron transport chain in relation to the Krebs cycle, the assumption being made that DPN-linked isocitric dehydrogenase partakes in this cycle. It can be seen that as oxidative phosphorylation proceeds, the concentration of ADP falls and that of ATP rises, while DPNH is being generated, but becomes immediately reoxidized to DPN^{+}, as long as electron transport occurs. The fact that DPN-linked isocitric dehydrogenase is, on the

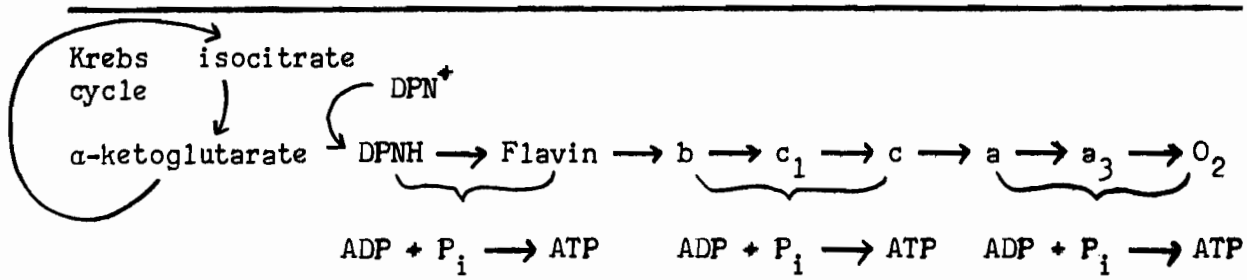


Fig. 21. The electron transport chain in relation to the Krebs cycle.

one hand, stimulated by ADP, and, on the other hand, inhibited by ATP and DPNH, suggests that the concentrations of these nucleotides might play a role in the regulation of the activity of the Krebs cycle and, hence, the oxidation of many substrates by mitochondria. Under conditions favoring respiration in mitochondria, it has been shown that the ratios, [DPNH]/[DPN$^+$] and [ATP]/[ADP], are low (see (177)). These conditions typify Chance's "State 3"; and under these circumstances, the oxidation of isocitrate by DPN-linked isocitric dehydrogenase would be favored since ADP is stimulatory and DPN$^+$ competitively counteracts the inhibitory effect of DPNH and ATP. Conversely, under anaerobic conditions, or where phosphate acceptor concentrations are low, the [DPNH]/[DPN$^+$] and [ATP]/[ADP] ratios are high ("State 4" of Chance). These conditions would predominate when there was excess substrate for oxidation but little demand for stored ATP. Under these conditions, DPN-linked isocitric dehydrogenase activity would be inhibited because the lack of ADP would diminish the affinity of the enzyme for isocitrate, and metal ions and the inhibition would be potentiated further by

the action of high concentrations of DPNH and of ATP at the site of DPN^+ binding. The inhibition by DPNH may be a highly effective mechanism for regulation of DPN-specific isocitric dehydrogenase under physiological conditions, because the apparent affinity of the enzyme for DPNH is markedly increased by TPNH. The extent of the inhibition of the enzyme by pyridine nucleotides would be a function not only of TPNH and DPNH, but also DPN^+. Alterations in the [ATP]/[ADP] and [DPNH]/[DPN^+] ratios in mitochondria could thus serve as a mechanism regulating the rate of substrate oxidation at the level of isocitrate. The potentiality for such regulation is present in DPN-linked isocitric dehydrogenase, and it is clear that the positive and negative feedback control exerted by nucleotides might turn the Krebs cycle oxidations on or off at just the right times.

One should recall that there is some evidence for some such regulatory mechanism, since a study of the oxidative metabolism of cardiac mitochondria showed that citrate oxidation was enhanced by lowering the ATP concentration and by addition of DPN^+ to the incubation medium (21). It might also be predicted that isolated mitochondria would exhibit different degrees of DPN-linked isocitric dehydrogenase activity depending on whether the mitochondria were aerobically or anaerobically incubated and how much endogenous substrate was present. Such differences in handling of mitochondria might explain the differences between the findings of Ernster's and Kaplan's laboratories concerning the presence or absence of the

enzyme in rat liver mitochondria (see Chapter I). In this regard, it may be significant that Krebs et al. (178) found that under anaerobic conditions, citrate reduced acetoacetate more rapidly than did α-ketoglutarate in rat liver homogenates. The authors concluded that "either transhydrogenase or DPN-linked isocitric dehydrogenase must be highly active". Under the experimental conditions used by these authors (178), DPNH would not accumulate since acetoacetate was added to reoxidize DPNH; and since the mitochondria of the homogenate were presumably tightly coupled, the anaerobic conditions would not permit ADP to be converted to ATP. Thus, ideal conditions would have existed to permit the action of DPN-linked isocitric dehydrogenase.

In connection with the possible regulatory role of nucleotides in cellular isocitrate oxidation, it is of interest that these studies have also shown that TPN-linked isocitric dehydrogenase of bovine heart is not influenced by ADP, ATP, DPN^+, or DPNH.

Concerning the problem of whether it is the TPN- or DPN-linked isocitric dehydrogenase which is present in the Krebs cycle, one might a priori tend to favor the DPN-linked enzyme since DPNH, rather than TPNH, is typically the coenzyme which is used in oxidation through the electron transport chain. It would seem unlikely that mitochondria would evolve with a cumbersome system of isocitrate oxidation which required first the formation of TPNH, then conversion of TPNH to DPNH via transhydrogenase, and finally oxidation along the electron transport chain. The function of TPN-linked isocitric

dehydrogenase might, on the other hand, be to oxidize isocitrate to yield TPNH for biosynthetic reactions. Many reactions in the field of fatty acid synthesis, mevalonic acid synthesis, steroid hydroxylation, tyrosine metabolism, and so forth, have been found to depend on TPNH and have been listed by Klingenberg and Bücher (179). Although these arguments concerning which enzyme actually is the Krebs cycle isocitric dehydrogenase are speculative, they are also bolstered by the fact that only the DPN-linked enzyme is definitely mitochondrial.

Furthermore, the finding that the DPN-linked enzyme seems suitable for a regulatory role in keeping with the energy needs of the cell again favors the idea that the enzyme plays an important role in oxidation. The fact that the TPN-linked isocitric dehydrogenase is present in such large amounts suggests that its main purpose is to reduce TPN^+ for biosynthetic reactions. Only a small amount of DPN-linked enzyme would be necessary, on the other hand, if it is the enzyme of the Krebs cycle, which is essentially catalytic in nature.

Since we have postulated that the DPN-linked isocitric dehydrogenase is subject to both positive and negative feedback control, one might wonder whether any other instances of such control are known. Frieden (180) has pointed out that glutamic dehydrogenase might be subject to both activation and inhibition in a way advantageous for the cell. Thus, α-ketoglutarate is produced by the enzyme from glutamate. When an excess of α-ketoglutarate is present, it

would be advantageous for the cell to inhibit glutamic dehydrogenase. This might be accomplished because the oxidation of α-ketoglutarate results in DPNH (which is inhibitory), which in turn is oxidized to yield ATP via oxidative phosphorylation (ATP is also inhibitory). Furthermore, the oxidation of α-ketoglutarate results in succinyl-CoA which reacts in a substrate-level phosphorylation to yield GTP (135), which is also inhibitory. Conversely, where there is insufficient substrate for oxidation, ADP might be high; and ADP stimulates glutamic dehydrogenase (89).

Another example of an enzyme subject to both positive and negative feedback control is the aspartate transcarbamylase of E. coli described by Gerhart and Pardee (118). This enzyme produces carbamylaspartate (ureidosuccinate), an early precursor of pyrimidines, which precedes the formation of dihydroorotic acid. This enzyme was found to be inhibited by a number of pyrimidines such as CTP but was stimulated by ATP. Thus, the synthesis of pyrimidines is slowed when there is an excess; but when there is an excess of purines, the synthesis of pyrimidines is speeded up in order to form nucleic acids as, for instance, during periods of rapid growth.

Although instances where both activation and inhibition of an enzyme occurs seem rare, there are many examples of purely negative feedback control. Thus, Umbarger (181) found that L-threonine conversion to L-isoleucine by E. coli was inhibited, probably at the L-threonine dehydrase level, by L-isoleucine. Formation of acetolactate, a precursor of L-valine, was inhibited by L-valine

(182). In L-proline biosynthesis by E. coli, a negative feedback has been postulated (183), and recently, Martin (171) showed that L-histidine inhibited its own biosynthesis at the level of the initial enzyme, phosphoribosyl-ATP pyrophosphorylase. Negative feedback control has been postulated for purine and pyrimidine biosynthesis; and, like the mechanism postulated involving DPN-linked isocitric dehydrogenase, some of these examples involve inhibition of enzymes by nucleotides. The activity of the enzyme, phosphoribosyl pyrophosphate-amidotransferase, may be regulated by inhibitory nucleotides such as ATP, ADP, GMP, AMP, and GDP (184). A negative feedback regulation of purine biosynthesis may also occur in Ehrlich ascites tumor cells, since Henderson (185) found that the extent of α-N-formyl glycinamide ribotide accumulation in azaserine-treated cells was inhibited by a number of purines and by 4-amino-5-imidazole carboxamide. A similar effect had been shown by Gots (186) to occur in E. coli, where adenine inhibited formation of the precursor, 4-amino-5-imidazole carboxamide. Inhibition by 5'-AMP and 5'-GMP of the reamination of IMP to 5'-AMP has been discussed in terms of a negative feedback mechanism (187). Pyrimidine biosynthesis in yeast and liver may be controlled by the concentration of various pyrimidine monophosphates, which inhibit the enzyme orotidylate decarboxylase (189,190). One may thus conclude that feedback control is not only fairly common, but a very probable mechanism of metabolic regulation.

The degree to which DPN-linked isocitric dehydrogenase is

actually controlled by pyrimidine and adenine nucleotides in mitochondria cannot be estimated with any certainty since the concentrations of these compounds have not been completely established. In addition, the levels of all these nucleotides would be expected to fluctuate in vivo. If mitochondria are isolated and the pyridine nucleotides measured, markedly different results are obtained depending on how well the mitochondria are shielded from air (191,192). However, several reports (192,193) indicate that cardiac mitochondria contain more of the oxidized nucleotides, DPN^+ and TPN^+, than the reduced nucleotides, DPNH and TPNH. Conversely, in liver mitochondria the reduced forms predominate. The fact that DPN-linked isocitric dehydrogenase activity is not favored by either DPNH or TPNH may explain why it is difficult to demonstrate the enzyme in liver mitochondria.

It should be pointed out that the regulatory mechanism postulated to work through DPN-linked isocitric dehydrogenase parallels closely the control of metabolic rates exerted by inorganic phosphate and phosphate acceptor systems. Since tightly coupled mitochondria do not oxidize substrates if phosphorylation cannot proceed due to insufficient ADP or P_i, the levels of these two compounds may determine the amount of oxidation which may occur. Thus, Lardy and Wellman (194) found that rat liver mitochondria oxidize citrate, pyruvate, α-ketoglutarate, and a number of other substrates only slowly; but oxidation increases markedly when any of the following are added: ADP, creatine and creatine-ATP

transphosphorylase, glucose and hexokinase, or 2,4-dinitrophenol. The assumption made was that each of these additions caused an increase in ADP concentration or else, in the case of 2,4-dinitrophenol, uncoupled oxidative phosphorylation, thereby allowing oxidation to proceed. However, all of these additions would also favor the activity of DPN-linked isocitric dehydrogenase, which is stimulated by ADP and inhibited by ATP. 2,4-Dinitrophenol activates mitochondrial ATPase (195), and would be expected to favor the activity of DPN-linked isocitric dehydrogenase.

In conclusion, it is hoped that these studies have helped to characterize DPN-linked isocitric dehydrogenase of bovine heart, and to elucidate some of its intrinsic properties which suggest that the enzyme may have an important role in mitochondrial oxidation.

CHAPTER VII

SUMMARY

DPN-linked isocitric dehydrogenase has been purified over 700-fold from bovine heart mitochondrial acetone powder. The purified protein exhibits a major component having a sedimentation constant of 10.3 S, and the molecular weight has been estimated to be about 3 or 4 x 10^5. The turnover number was calculated to be about 8000 moles of DPNH formed per minute per mole of enzyme.

ADP has been found to affect this enzyme in several ways. The nucleotide stabilizes the enzyme under conditions of low ionic strength. ADP also enhances the activity of the enzyme, and this effect has been found to be due to a marked diminution of the K_m for isocitrate. In addition, K_m for metal ions is also reduced by ADP. At low isocitrate concentrations, such as may exist in mitochondria, the enzyme is virtually dependent on ADP for activity. The activating effect of ADP is highly specific since, of a large number of nucleotides tested, only ADP and dADP are stimulatory. In the presence of low concentrations of isocitrate, the pH optimum is displaced from pH 6.7 in the absence of ADP to about pH 7.2 in the presence of ADP. An increase of substrate concentration in the absence of ADP leads to a similar shift of the pH optimum.

The enzyme is inhibited by DPNH, ATP, and ADPR; the inhibition

is competitive with DPN^+. TPNH potentiates the DPNH inhibition, and both TPNH and DPNH apparently can form fluorimetrically discernible complexes with the enzyme. On the other hand, the TPN-linked isocitric dehydrogenase of bovine heart has been found to be insensitive to DPN^+, DPNH, ATP, and ADP.

The mechanism of activation by ADP is probably a conformational change in the enzyme which results in the active site becoming more accessible to substrate. In the ultracentrifuge, it has been shown that the sedimentation velocity of the enzyme is markedly increased by ADP, a finding which suggests that aggregation has occurred.

The significance of these findings has been discussed in terms of a possible positive-plus-negative feedback control mechanism for mitochondrial oxidation.

In addition, stereospecific syntheses of threo-D_s-isocitrate-α-T and of threo-D_s-isocitrate-β-T have been accomplished enzymically. Oxidation of these compounds by DPN-linked isocitric dehydrogenase revealed that the α-hydrogen of isocitrate was transferred stereospecifically and directly to the α-side of DPN^+, and that the β-hydrogen of isocitrate was retained in α-ketoglutarate. The β-hydrogen was also retained during the reaction with TPN-linked isocitric dehydrogenase, thus indicating that the enol form of oxalosuccinate is not likely to occur as a free intermediate in the oxidation of isocitrate. Thus, in all respects, the hydrogen transfer mediated by DPN-linked isocitric dehydrogenase was the same as that catalyzed by the TPN-specific enzyme.

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